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"THE ISOLATION, PURIFICATION, AND CHARACTERIZATION OF A BACTERIOPHAGE INFECTIOUS FOR PSEUDOMONAS FLUORESCENS".

BY

D. WARK BOUCHER

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "The Isolation, Purification, and Characterization of a Bacteriophage Infectious for Pseudomonas fluorescens, submitted by D.Wark Boucher,B.Sc., in partial fulfilment of the requirements for the degree of Master of Science in Microbiology.

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ABSTRACT

The isolation of several pseudomonas phage from sewage is described. Two of these, designated as phage 16 and phage B, were chosen for further study. Both phage had a similar host range but could be differentiated on the basis of plaque morphology.

Purification of the phage on DEAE cellulose was studied. Phage 16 could be purified on DEAE cellulose. The phage were eluted from the column at a NaCl concentration of 0.2M. The purity of the preparation obtained by column chromatography was comprable to that obtained on a CsCl gradient. The ratios of the optical density at 230 mu to the optical density at 260 mu (O.D.230/O.D.260) and the optical density at 260 mu to the optical density at 280 mu (O.D.260/O.D.280) were used as a measure of the degree of purity of the phage preparation. Attempts to purify phage B on DEAE cellulose were unsuccessful.The phage did not adsorb on the cellulose and 99% of the infectious phage were lost.

The one step growth curves for phage 16 and phage B are discussed. Initial results indicate that the phage have longer latent and rise periods than the T-even phage of E. coli. An interesting feature of phage 16 and phage B is the failure of the phage to cause clearing of a broth culture of susceptible bacteria.

Phage 16 was found to contain deoxyribonucleic acid (DNA). Chemical and physical data indicate that the

DNA contains the four normal bases adenine, guanine, cytosine, and thymine. No glucose or unusual bases were found. The phage DNA contains 43 mole percent guanine plus cytosine.

The molecular weight of phage 16 calculated from its diameter (Adams, 1959) was found to be approximately 59×10^6 molecular weight units. The molecular weight of phage 16 DNA from phosphorus measurements was approximately 36×10^6 molecular weight units.

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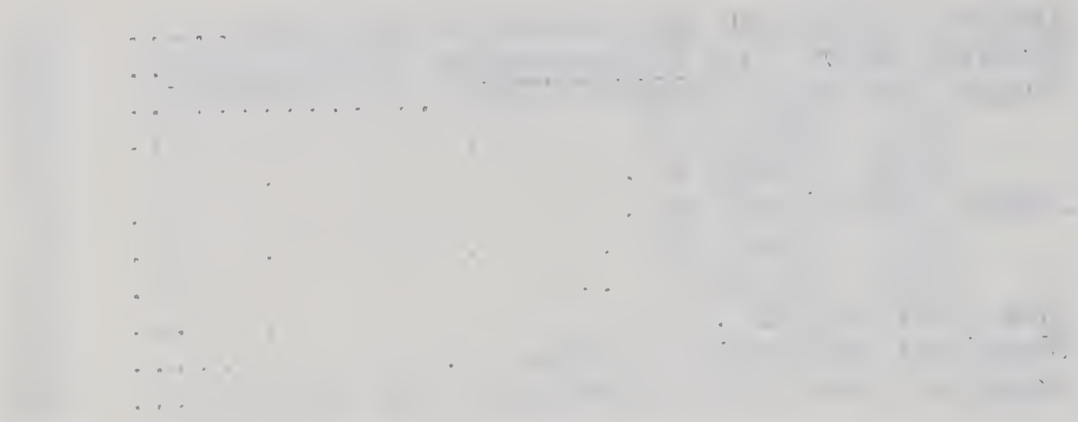

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INTRODUCTION

Phage infectious for pseudomonas were first reported in 1923 by Cancik; Combiesco and Margheru; and Pons. The studies done at that time and since then have been concerned mainly with lysogeny and the use of lysogeny in grouping pseudomonas (Hadley, 1924; Rabinowitz,1934; Fastier, 1945; Warner, 1950; Holloway,1960a; Holloway etal, 1960b; Feary etal 1963a).

In one of the first reports, Hadley (1924) described "pitted or pocketed" areas, one to four mm. in diameter on the surface of bacterial growth. These areas appeared to extend down to the agar substratum. He found that this phenomenon was not only transmittable from the clear area to a subculture but also from the growth surrounding these clear areas. Hadley concluded that the factor causing lysis was not a substance foreign to the cell, but a normal part of the cell organization which was stimulated by an unrecognizable agent.

Holloway etal (1960,b) and Feary etal (1963,a) have since shown that patterns of lysogenicity obtained in relation to a set of indicator strains, can serve as a useful method for grouping strains of Pseudomonas aeruginosa. By using lysogeny and pyocinogenicity, Holloway (1960,a) was able to place 214 strains of Pseudomonas aeruginosa into 18 groups.

Work dealing specifically with the growth character and nucleic acid composition of a phage infectious

for pseudomonas was not reported until 1963 (Feary etal. 1963,b). Feary etal. (1964) found that phage 7v, isolated from a lysogenic strain of Pseudomonas aeruginosa, had a latent period of approximately 20 minutes, a rise period of approximately 10 minutes, and a burst size of 200. The nucleic acid of a second phage designated as 7s, was found to be RNA (Feary etal. 1963,b).

In comparison, the T series of E. coli phage have been well characterized (Adams, 1959; Sinsheimer, 1960; Stent, 1963). The single step growth curves for these phages are approximately the same. At 37°C the latent period is 20 to 25 minutes and the rise period is approximately 10 minutes. All the T phage contain DNA as their nucleic acid (Stent, 1963. Table 3-111). The most interesting feature of this group is the fact that the T even phage contain 5-hydroxymethylcytosine (Wyatt and Cohen 1952) and glucose (Sinsheimer, 1954; Volkin, 1954; Jesaitus, 1956) in their nucleic acid. The T odd phage, T1, T3, T5, and T7 contain the four usual bases of DNA, adenine, thymine, guanine, and cytosine (Sinsheimer, 1960; Stent, 1963).

In addition to the T phage other phage have now been discovered which also contain unusual bases in their nucleic acid. Kallen etal. (1962) showed that a phage infectious for Bacillus subtilis contained 5-hydroxymethyluracil and Takahashi and Marmur (1963) isolated another phage infectious for Bacillus subtilis which contained glucose and uracil in the DNA.

It was of interest therefore to see how phage

infectious for pseudomonas compared with other phage, particularly those infectious for E.coli. At the time this research was undertaken there were no reports in the literature on the growth character or nucleic acid content of a DNA phage infectious for pseudomonas. Since then Grogan and Johnson (1964,a 1964,b) and Niblack and Gunsalus (1965) have reported on phage infectious for pseudomonas which contain DNA. Their findings indicate that these phage contain adenine, thymine, guanine and cytosine in the DNA. No unusual bases or glucose were present. The amount of guanine plus cytosine however was found to be greater than in the T phage of E.coli.

The purpose of this study is to investigate the growth and nucleic acid content of a phage infectious for Pseudomonas fluorescens. The phage, chosen from a number isolated in this laboratory, could be purified easily on a cellulose column. Both chemical and physical measurements were employed in characterizing the nucleic acid.

MATERIALS AND METHODS

1. Bacterial Strains.

A total of 50 strains of pseudomonas were used as host organisms for the isolation of phage from sewage. Of these, 26 were obtained from laboratory stock cultures and 24 were obtained from the Provincial Laboratory of Public Health in Edmonton, Alberta. The latter strains were isolated from specimens at the Provincial Laboratory and will be given the prefix PL.

2. Maintenance and Growth of Bacteria.

The pseudomonas strains were maintained as stock cultures on Difco Trypticase Soy slants at 4°C. The stock cultures were transferred to fresh media every 4 weeks. When bacterial strains were required they were transferred as innoculum into Trypticase Soy broth or nutrient broth and incubated overnight at 37°C on a Burrell wrist action shaker.

3. Isolation of Phage from Sewage.

At the time this study was undertaken little work had been done on a pseudomonas phage in this laboratory. It was necessary therefore to isolate phage for further study.

Raw sewage, obtained from the city of Edmonton sewage plant, was allowed to stand overnight at 4°C to enable the heavy sludge to settle out. To remove bacteria and debris the supernatant was centrifuged for 15 minutes at 10,000 rpm. in a SS 34 rotor of the Servall RC-2 centrifuge.

The probability of phage detection was improved

if the supernatant was centrifuged for 90 minutes at 18,000 rpm. in a SS 34 rotor of the Servall RC-2 centrifuge. This concentrates most of the phage which may have been present. The pellet resulting from centrifugation was resuspended in 5 ml. of 0.04M PO_4 buffer at pH 7.0. This represented a concentration of approximately 15 times that of the original preparation.

4. Assay of Sewage.

Assay plates for phage detection were seeded with the 50 strains of pseudomonas by mixing 2 drops of an overnight bacterial culture with 2 ml. of melted soft nutrient agar. The mixture was poured on nutrient agar plates and after it had hardened 2 drops of the sewage was placed on the agar and spread with a glass rod. The plates were examined for plaques after overnight incubation at 30 or 37°C.

5. Isolation of Pure Phage.

To obtain a single plaque type of phage, isolated plaques were picked with an innoculating needle, spotted on a bacterial lawn, and spread with a glass rod. After overnight incubation at 37°C isolated plaques were picked and the procedure was repeated until it was certain a single plaque type was being propagated.

Once a single plaque type had been isolated several plates of the phage were grown and harvested. This served as a stock of the phage.

Phage were harvested by the method described in Adams (1959, pp 456). Ten mls of nutrient broth were

poured on the plate. The soft agar layer and nutrient broth were scraped into a beaker and mixed on a magnetic stirer for 20 minutes to elute the phage from the agar. To remove the bacteria and agar the mixture was centrifuged for 10 minutes at 10,000 rpm. in a SS 34 rotor of the Servall RC-2 centrifuge.

6. Preparation of High Titre Phage Solutions.

It is essential in phage studies that one have a preparation with as high a titre as possible. In the method to be described a titre of 1×10^{12} per 0.1 ml is usually obtained.

The agar layer method described by Hershey etal. (1943) and outlined by Adams (1959, pp.457) was found to give the best results. Two drops of an overnight bacterial culture were mixed in soft nutrient agar with enough phage to give confluent lysis on the plates. The mixture was poured on a nutrient agar plate and incubated overnight at 37°C. The plates were harvested as described (page 5). The supernatant obtained after centrifugation at 10,000 rpm. was centrifuged for 30 minutes at 25,000 rpm. in a type 30 rotor of the Beckman model L-2 ultracentrifuge. The pellets were resuspended to one-tenth the original volume in 0.04M PO_4 buffer, pH 7.0. This was the phage preparation used for further analysis.

7. Host Range and Plaque Morphology.

Initial characterization of phage can be done on the basis of plaque morphology and host range.

To study the host range, bacterial lawns of the 50 pseudomonas strains were prepared as described (page 5). After the lawn had hardened, a drop of the phage suspension

was spread on the plate. This suspension contained enough phage to give 100 to 200 plaques on the strain from which it was isolated. Since quantitation was not desired, the bacterial strains were scored as sensitive if plaques were produced, and insensitive if no plaques were produced.

The plaque morphology of the phages was studied by serially diluting the phage stocks so that 0.1 ml of the phage suspension would give 10 to 50 plaques when plated by the agar layer technique. After overnight incubation the plates were examined for the type of plaque produced by the different phage on each strain of bacteria.

8. Bacterial Growth Curve.

In order to study the phage single step growth curve, it was necessary to determine when the bacteria entered the log phase of growth, and to correlate bacterial titres with optical density, under defined conditions.

A flask containing 100 ml of nutrient broth was inoculated with an overnight culture of Pseudomonas fluorescens strain OAC 99. The optical density at 600 mμ was adjusted to read 0.1 on a Bausch and Lomb Spectronic 20. The flasks were placed in an Eberbach constant temperature water bath and agitated at 120 cycles per minute at 37°C. The optical density at 600 mμ was determined every 15 minutes on the Bausch and Lomb Spectronic 20. The number of viable cells was determined by serially diluting the culture and mixing 0.1 ml of the desired dilution with 2 ml soft nutrient agar. The mixture was plated on nutrient

agar plates and incubated overnight at 37°C. The colonies were counted on a New Brunswick Scientific Counter.

9. Phage Single Step Growth Curve

To study the single step growth curve, 100 ml. of Pseudomonas fluorescens at a density of 1 X 10^8 per ml., were inoculated with phage at a multiplicity of 1 and 10. The phage suspension used contained 1 X 10^{13} phage per ml.. The mixture was incubated for 10 minutes at 37°C, with aeration, to allow adsorption of the phage. The mixture was then serially diluted, in 9 ml. nutrient broth blanks, to 10^{-8}. The last three dilutions, 10^{-6}, 10^{-7}, and 10^{-8} were incubated with aeration at 37°C. These dilutions were assayed for infectious phage every 15 minutes by taking 0.1 ml. aliquots and plating by the agar layer technique.

10. Phage Purification

(1) Initial

Initial purification and concentration of the phage consisted of differential centrifugation. Phages obtained from harvesting agar plates were centrifuged for 30 minutes at 25,000 rpm. in a type 30 rotor head of the Beckman model L-2 ultracentrifuge. The resultant pellet was resuspended in 50 ml. of 0.04M PO_4 buffer, pH 7.0 and the solution was centrifuged at 10,000 rpm. for 10 minutes. The supernatant was then centrifuged for 30 minutes at 25,000 rpm. to sediment the phage and the pellet was resuspended in 50 ml. of 0.04M PO_4 buffer, pH 7.0.

(11) Column Chromatography

For further purification 15 mm. DEAE cellulose was placed in a column measuring 5 mm. by 62 mm. and phage were adsorbed on the cellulose. The column was washed once with 100 mls. of 0.04M PO_4 buffer, pH 7.0 followed by 100 ml. increments of increasing NaCl solutions in 0.04M PO_4 buffer, pH 7.0. (The NaCl solutions used contained 0.05M, 0.1M, 0.2M, 0.3M, 0.4M and 0.5M NaCl.)

The absorption spectrum of each fraction from 200 mμ to 300 mμ was recorded on a Bausch and Lomb Recording Spectrophotometer, model 505. The purity of the solution was determined from the ratio of the optical density at 230 mμ to the optical density at 260 mμ (O.D.230/O.D.260) and the optical density at 260 mμ to the optical density at 280 mμ (O.D.260/O.D.280). The titre of each fraction was also recorded.

(111) Enzyme Treatment

The fraction from the column containing the phage was centrifuged for 30 minutes at 30,000 rpm. on the Beckman model L-2 ultracentrifuge. The pellet of phage was resuspended in 0.04M Tris buffer (2-amino-2-hydroxymethylpropane-1:3-diol) pH 7.0.

The preparation was treated with 10 ug per ml of RN ase for 60 minutes at 37°C, pH 7.6 (Grade A ribonuclease, 5X crystalized, obtained from Cal biochem was used.) $MgSO_4$ was then added to a final concentration of 0.003M followed by addition of DN'ase

to a final concentration of 10 µg per ml. (Grade A DN ase, 1X crystalized, obtained from Worthington Biochemical Corporation was used.) Following a 60 minute incubation period at 37°C the solution was centrifuged for 30 minutes at 25,000 rpm. in a type 30 rotor of the Beckman model L-2 ultracentrifuge.

The phage were resuspended in either 0.04M PO_4 or 0.04M Tris buffer, pH 7.0. The titre of the solution was approximately 1 X 10^{13} per ml.

(1V) CsCl density gradient

As a means of further purification the phage solution was centrifuged on a preformed CsCl gradient. A 0.8 ml phage suspension purified on DEAE cellulose and containing approximately 5 X 10^{13} phage per ml. was placed on a preformed CsCl gradient. The phage were suspended in 0.05M Tris buffer, pH 7.0. The gradient was prepared by successive layering of 0.7 ml. of 5 CsCl solutions whose densities were 1.62 gm.cm.$^{-3}$, 1.49 gm.cm.$^{-3}$, 1.44 gm.cm.$^{-3}$, 1.33 gm.cm.$^{-3}$, and 1.22 gm.cm.$^{-3}$. The preparation was centrifuged for 45 minutes at 25,000 rpm. in a swinging bucket rotor SW 39 of the Beckman model L-2 ultracentrifuge. After the rotor head had come to a stop unbraked, the tubes were secured in a vertical position. After pinhole perforation of the bottom of the tube, four drop fractions were collected. The density of each fraction was measured by weighing fixed volumes in a lambda pipette. Each fraction was assayed for phage and the absorption spectrum from 200 mµ to 300 mµ was

recorded on a Bausch and Lomb Recording Spectrophotometer model 505.

(V) Other

Attempts to purify phage on sephadex gel, ECTEOLA or by precipitation with ethanol or $(NH_4)_2SO_4$ were also tried.

11. Isolation of DNA

For studies on DNA it is necessary to separate the DNA from the protein shell of the phage and in the case of bacteria from the cell components.

The most satisfactory method of preparing the phage DNA was found to be the phenol extraction procedure of Mandell and Hershey (1960). The criterion of a good preparation was taken to be the O.D.230/O.D.260 and O.D.260/O.D.280 ratios. The ratios were obtained from the absorption spectrum of the DNA solution recorded from 200 mμ to 300 mμ on a Bausch and Lomb Recording Spectrophotometer model 505.

DNA was isolated from phage purified on a CsCl gradient or by elution from DEAE cellulose. Five mls. of phage suspension with a titre of 1×10^{13} per ml and suspended in 0.1M NaCl plus 0.04M Tris buffer, pH 7, were mixed with an equal volume of phenol on a Fisher mini-shaker for 10 minutes. The emulsion was separated into three layers by a 10 minute centrifugation at 7,000 rpm. The upper layer containing the nucleic acid was removed with a pipette, transfered to a test tube and washed five times with an equal volume of ether. Traces of

ether were removed by bubbling air through the mixture. Finally a 0.5 volume of isopropanol was layered on the DNA solution. The two layers were mixed with a glass stirring rod and the DNA was wound on the rod. The DNA fibres were washed in 70% ethanol and then redissolved in 0.0015M Tris buffer, pH 7.1.

DNA was isolated from bacteria by the procedure of Marmur (1961). Five hundred mls. of an overnight broth culture of bacteria were centrifuged for 10 minutes at 15,000 rpm. in a SS 34 rotor of the Servall RC 2 centrifuge. The pellet of bacteria was washed in 100 ml. of 0.15M NaCl plus 0.1M ethylenediaminetetra-acetate (EDTA), pH 8 and centrifuged again at 15,000 rpm. for 10 minutes. To lyse the cells the bacteria were suspended in 15 ml. saline-EDTA, to which was added 1 ml. sodium lauryl sulfate ($NaC_{12}H_{26}SO_4$). The mixture was then placed in a 60°C water bath for 10 minutes. The lysate was cooled, perchlorate added to a final concentration of 1M, and the mixture shaken for 10 minutes with an equal volume of phenol on a Fisher mini-shaker. The emulsion was separated into three layers by centrifugation at 7,000 rpm. for 10 minutes. The nucleic acid was removed and the procedure was repeated until no protein appeared at the interphase. The nucleic acid was then treated with ether etc. as previously described in the isolation of DNA from phage.

The fibres of DNA were redissolved in

0.15M NaCl plus 0.015M trisodium citrate, pH 7.0. RN'ase was added to a final concentration of 10 µg per ml. and the solution was incubated for 30 minutes at 37°C. The DNA was again precipitated with 0.5 volumes of isopropanol, washed in 80% ethanol, and dissolved in 0.015M NaCl plus 0.0015M trisodium citrate.

The purity of the DNA was determined by its absorption spectrum from 200 mµ to 300 mµ.

12. Hydrolysis of DNA

Chemical analysis of phage DNA and bacterial DNA required the breakdown of the nucleic acid to the constituent purine and pyrimidine bases.

The best technique for obtaining free bases from DNA was found to be that of Wyatt and Cohen (1953). One ml. of a DNA preparation containing approximately 1 mg. of DNA per ml. was mixed with 0.5 ml. formic acid in a pyrex glass tube with an internal diameter of 6 mm. The tube was sealed and the mixture heated at 175°C for 30 minutes. The tube was carefully opened and the hydrolysate taken to dryness in vacuo over P_2O_5 at room temperature. The residue was dissolved in 20λ 0.1N HCl; one 10λ portion was chromatogramed, the other 10λ portion was used for phosphorus analysis.

13. DNA Analysis

(1) Physical

The density, in CsCl, of DNA'S from various sources have been shown to be linearly related to the mole per cent guanine plus cytosine (G + C) Sueoka etal.

1959; Rolfe and Meselson, 1959; Schildkraut etal. 1962). The amount of guanine plus cytosine as found by the buoyant density of DNA in CsCl was determined for a comparison with chemically determined values.

CsCl suspended in 0.005M Tris buffer plus 0.005M EDTA, pH 8.1, was mixed with the different DNA samples (Marmur, 1961). The density of the CsCl DNA solution was adjusted to 1.72 $\pm$ 0.002 gm. cm.$^{-3}$ by weighing fixed volumes in a lambda pipette. The mixture was centrifuged at 20°C for 48 hours at 30,000 rpm. in a swinging bucket rotor SW 39 of the Beckman model L-2 preparative ultracentrifuge. The rotor head was allowed to come to a stop unbraked, the tubes were placed in a vertical position and four drop fractions were collected after pinhole preforation of the bottom of the tube. The density of each fraction was measured by weighing fixed volumes in a lambda pipette. To determine which fraction contained the DNA the absorption spectrum of each fraction was recorded on a Bausch and Lomb Recording Spectrophotometer model 505 from 200 mμ to 300 mμ.

(11) Chemical

Paper chromatography was used to separate the bases obtained by acid hydrolysis. All chromatograms were run on Whatman No.1 chromatography paper, 27 cm. long and 18 cm. wide. Two solvent systems were used, separately in one dimensional chromatography, and together in two dimensional chromatography.

(a) Isopropanol - HCl (PrH) (Wyatt, 1951)

isopropanol	130 ml.
H_2O	37 ml.
HCl	33 ml.

(b) Butanol ammonia (Wyatt, 1955)

isobutyric acid	100 ml.
H_2O	55.8 ml.
ammonia .88	4.2 ml.
versene 0.1M (37 g/l)	1.6 ml.

The final pH of the solvent should be 4.6

Ten lambda of the redissolved hydrolysate was applied at one end of the filter paper and one dimensional chromatography was carried out at 28°C with isopropanol-HCl as the solvent. The end of the filter paper near the spot was immersed in a trough of solvent; the other end hung free in an enclosed glass jar so that the atmosphere was saturated. Since the solvent was allowed to run off the paper, the free edge was serrated so that pulling or tailing to one side would not occur if the solvent front ran unevenly. At the end of 38 hours the paper was removed, dried, and the spots were located with a General Electric ultraviolet hand lamp.

Under ultraviolet light the purine and pyrimidine derivatives generally appear as dark spots against a light blue fluorescence of the paper. When an acid solvent is used, especially HCl, guanine and xanthine and their compounds fluoresce quite strongly. (Smith, 1960).

A permanent record of the bases was obtained by placing a chromatogram over Kodak Kodabromide F-5 paper and exposing both to a 1 second flash of ultraviolet light. When the print is developed, the bases will appear as light areas on a black background since they absorb the ultraviolet light.

Two dimensional chromatograms were run with isopropanol-HCl as the solvent in the first dimension and butanol-ammonia as the solvent in the second dimension. The chromatograms were allowed to run for 18 hours in the first dimension before being removed and dried at room temperature. A strip was sewn on the bottom of the portion containing the partially separated bases and the second dimension was run for a further 24 hours before being removed and dried.

After the bases had been located the percentage of each base in the DNA was determined as follows. For quantitative estimation the spots were cut out and eluted in 5 mls. of 0.1N HCl in a stoppered test tube. To allow for ultraviolet absorbing substances in the paper, identical pieces were cut from a corresponding position in a blank lane and treated the same as the bases. After overnight incubation at 37°C the tubes were shaken and the absorption spectrum of the eluant recorded from 200 mμ to 300 mμ on a Bausch and Lomb Recording Spectrophotometer model 505. The concentration of each base was determined from absorption maxima extinctions (Bendich, 1957).

Since the nucleic acid of some other phages

contain glucose (Sinsheimer, 1954; Jesaitus, 1956) tests were done to determine if glucose was present in the phage studied. The technique used was adapted from Takahashi and Marmur (1963). The purified DNA was hydrolysed in formic acid as described and taken to dryness. The residue was resuspended in 20 λ of 1N HCl. The entire amount was then spotted on the chromatogram. Isopropanol-HCl was used as a solvent in one dimensional chromatography. The chromatogram was allowed to run for 38 hours before being removed and dried. Aniline diphenylamine reagent (aniline, 1 percent (1 ml.), plus diphenylamine, 1 percent in acetone 10 volumes plus H_3PO_4; 1 volume) was used to locate the reducing sugar glucose. A control solution was treated in the same manner and compared with the DNA.

If one determines the mole percent phosphorus liberated by acid hydrolysis it is possible to calculate the percent recovery of the bases and the approximate molecular weight of the DNA (Stent 1963, p. 65).

Phosphorus was determined by the method of Allen (1940). Ten lambda of the hydrolysed DNA were placed in a micro-Kjeldhal flask with 2.2 ml. perchloric acid. the flask was heated on a Lab Con model A micro-burner until the contents became colorless. The mixture was cooled and rinsed into a 25 ml. volumetric flask. To the mixture was added 2 ml. amidol, 1 ml. molybdate and water to 25 ml.. The color was allowed to develop for 15 minutes and then the optical density was read at 550 mμ on a Bausch and Lomb Spectronic 20. The amount of phosphorus was found from a

standard curve on which the optical density was plotted against mg. of phosphorus.

RESULTS

1. Phage Isolation and Characterization.

Of the 50 pseudomonas strains used as hosts only 9 had visible plaques after being exposed to the sewage filtrate. In some instances one bacterial strain gave rise to more than one type of plaque. Table 1 lists the different strains which showed plaques together with the morphology of the plaques.

When the different plaque types were tested on the other bacterial strains which had given rise to plaques, it was apparent that the two plaque types isolated on P. fluorescens strain OAC 99, phage A and phage B, were the same as those isolated on P. fluorescens strain PAE 2-1. The evidence for this was based on the similarity in the plaque morphology (table 2) and host range (table 3) of the phage when plated on various different strains.

Of the other phage isolated the phage isolated on PL 3584 was the same as the phage isolated on PL 3490.

Of the phage isolated two, designated as phage B and phage 16, were chosen for further study. Pseudomonas fluorescens strain OAC-99 was chosen as the host since both phage types appeared to multiply best on this strain. Phage 16 gave very small diffuse plaques which varied in size (plate 1) while phage B gave large distinct plaques with a halo (plate 2). The host range for the two phage is the same (table 4).

TABLE 1.

Plaque Type Obtained on Pseudomonas Strains

Strain	Plaque Morphology
PL 3490	distinct plaques, no halo or diffuse area, size 2.5 mm. in diameter
PL 6094	distinct plaques, no halo or diffuse area, size 2.5 mm. in diameter
PL 3584	distinct plaques, no halo or diffuse area, size 2.5 in diameter
PL 6375	clear area with halo, size 2mm. in diameter
PL 3705	Ø A small distinct clear area, size 2 mm. in diameter Ø B very small diffuse plaques
27'	small diffuse plaques, no halo
MAC 264	small clear plaques, no halo
OAC 99	Ø A small plaques, no halo Ø B large clear area, with halo, size 2 mm. in diameter
PAE-2-1	Ø A clear plaque, no halo, size 2 mm. in diameter Ø B large clear plaque with halo, size 3 mm. in diameter

TABLE 2.

Host Range of Phage Isolated on *Pseudomonas fluorescens* Strains OAC 99 and PAE 2-1

Indicator* Strains	Phage Isolated on OAC 99		Phage Isolated on PAE 2-1	
	A	B	A	B
OAC 99	+	+	+	+
PAE 2-1	+	+	+	+
MAC 264	+	+	+	+
139	-	+	-	+
124	-	+	-	+
20	-	+	-	+

* OAC 99, PAE 2-1 — *P. fluorescens*

MAC 264, 139, 124, 20 — *P. aeruginosa*

TABLE 3.

Plaque Type of Phage Isolated on
Pseudomonas fluorescens Strains OAC 99 and PAE 2-1

Indicator Strain	Phage Isolated on OAC 99		Phage Isolated on PAE 2-1	
	A	B	A	B
OAC 99	small plaque, no halo	clear area with halo, total diameter 3 mm.	small plaque, no halo	clear area with halo, total diameter 3 mm.
PAE 2-1	large plaque, no halo, diameter 1 mm.	clear area with halo, total diameter 3 mm.	large plaque, no halo, diameter 1 mm.	clear area with halo, total diameter 3 mm.
MAC 264	small pinpoint plaque	clear area with halo, total diameter 3 mm.	small pinpoint plaque	clear area with halo, total diameter 3 mm.

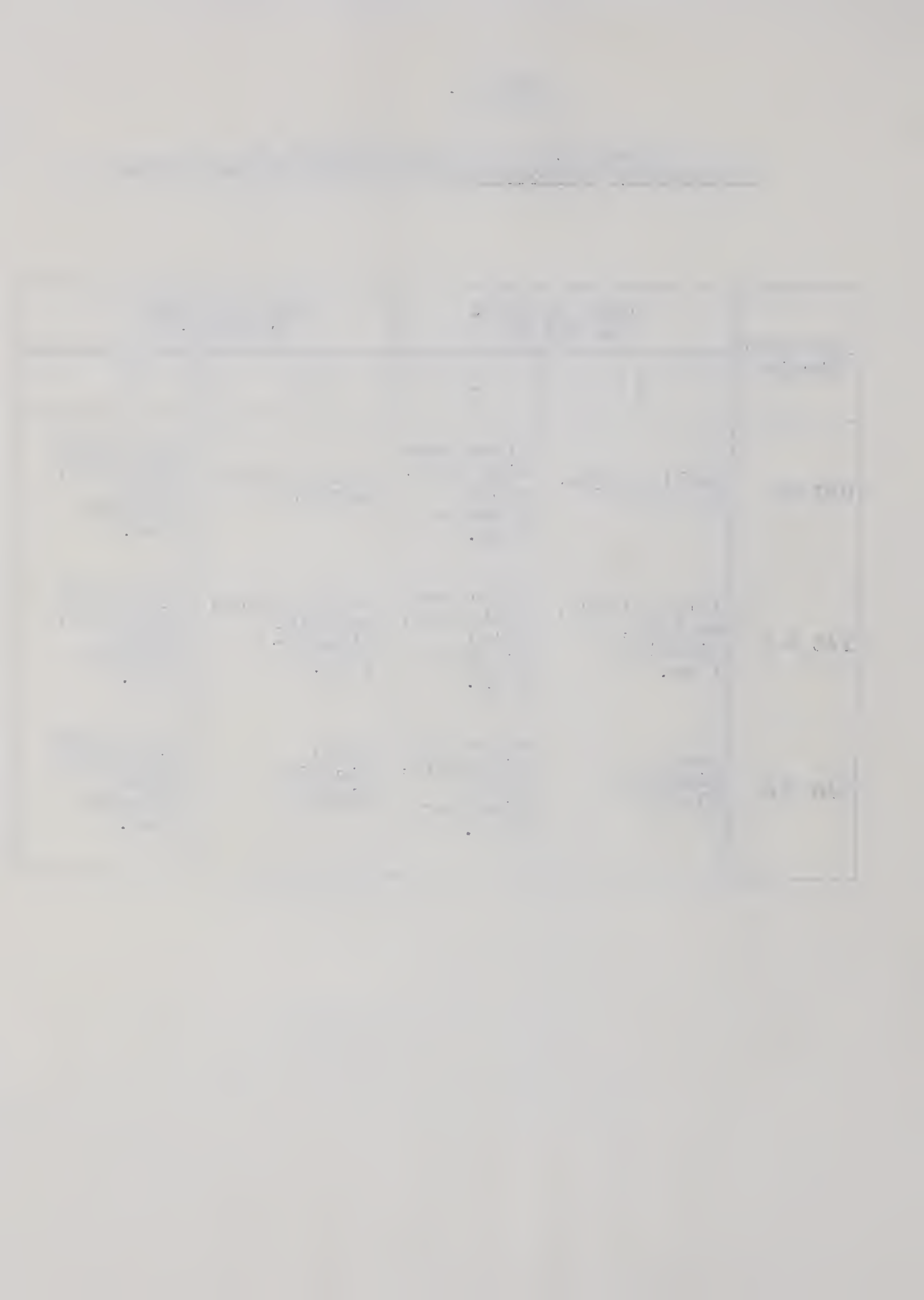


PLATE 1.

Phage 16 Plaques

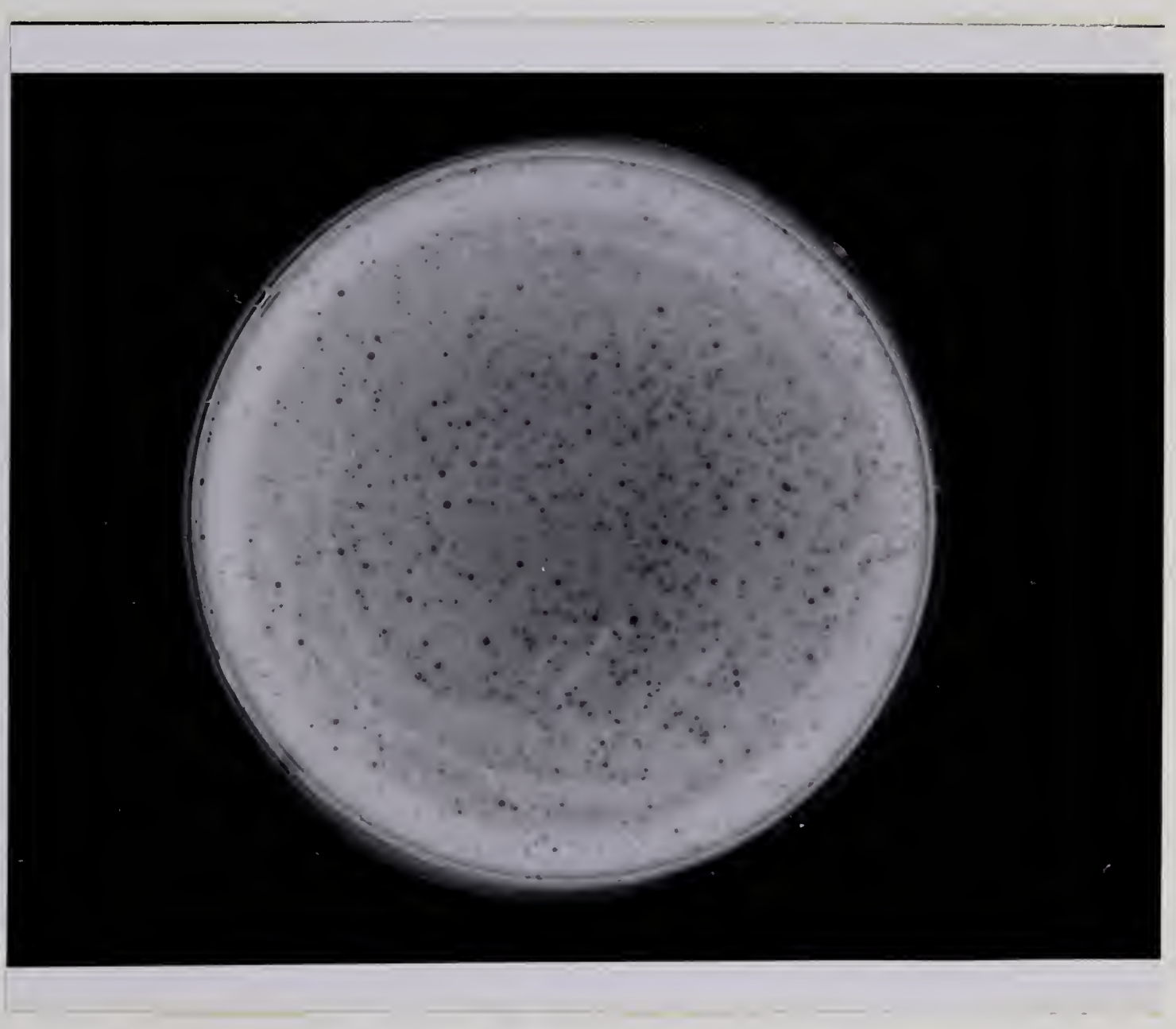


PLATE 2.

Phage B Plaques

TABLE 4.

Phage B and Phage 16 Host Range

Bacterial Strains	Phage	
	B	16
OAC 99	+	+
PAE 2-1	+	+
MAC 264	+	+
139	+	+
24	+	+
20	+	-

2. Growth Curve for Pseudomonas fluorescens Strain OAC 99

The result of the growth curve for P. fluorescens is illustrated graphically in figure 1. After nutrient broth was inoculated with an overnight culture of OAC 99 and the optical density adjusted to 0.1 at 600 mu, there was a lag period of one hour before the bacteria entered the logarithmic phase of growth. Since phage were introduced into the media near the start of the log phase, the growth curve was not continued into the statioary phase of bacterial growth.

Since an approximation of the number of viable cells was needed for the phage one step growth curve, a curve plotting optical density versus viable cells was drawn (figure 2). This made it possible to estimate the number of viable bacteria directly from the optical density.

3. Phage One Step Growth

(1) Phage 16:

An overnight culture of P. fluorescens was inoculated into nutrient broth and the optical density was adjusted to 0.1 at 600 mu. The preparation was incubated at 37°C for one hour on an Eberbach water bath shaker. The number of viable cells after incubation was estimated from the optical density at 600 mu, and phage 16 was introduced into the mixture at a multiplicity of 1 and 10. The phage were allowed to adsorb to the bacteria for 10 minutes and then the mixture was serially diluted. The actual number of phage and bacteria was

Figure 1

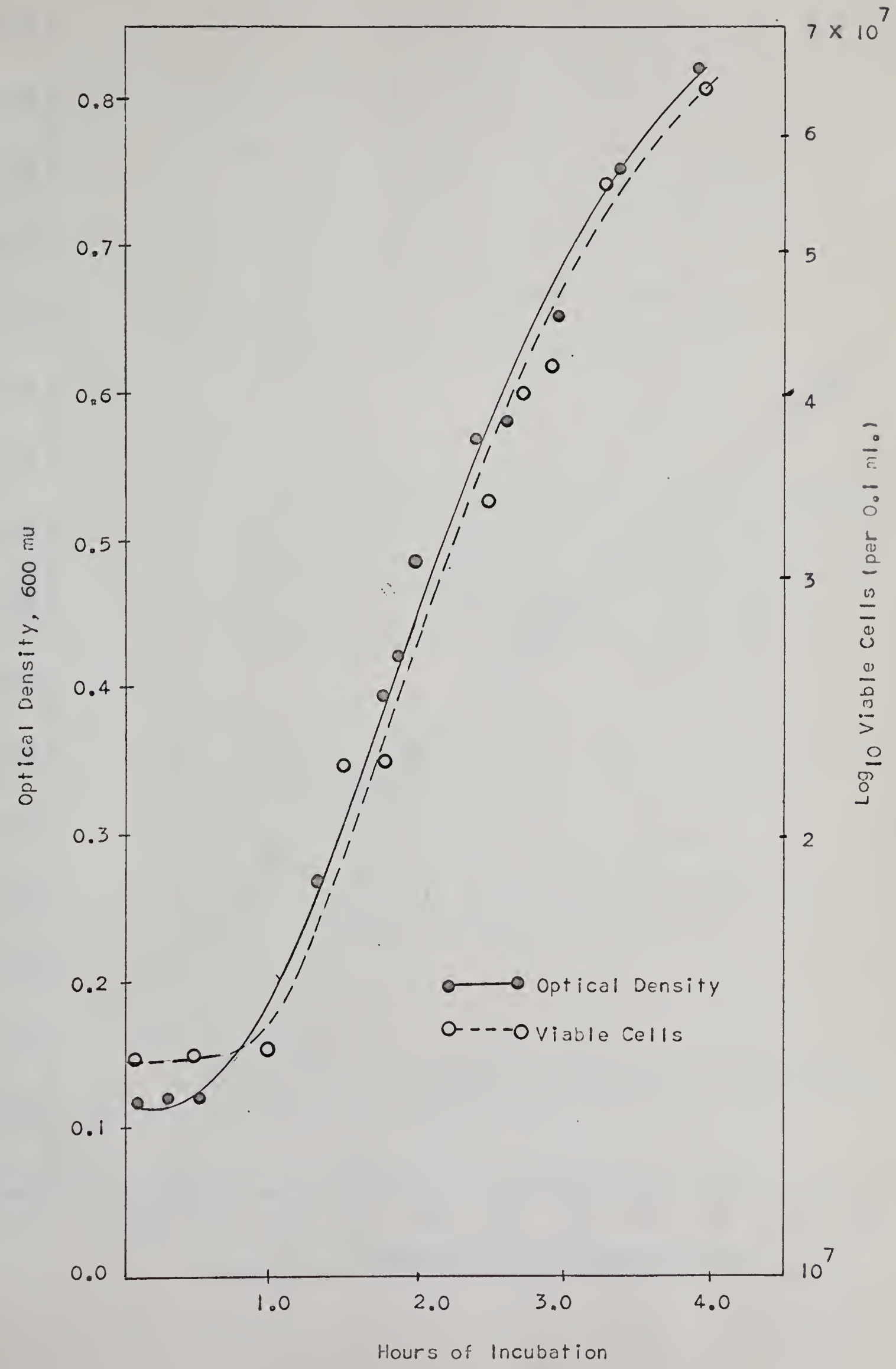
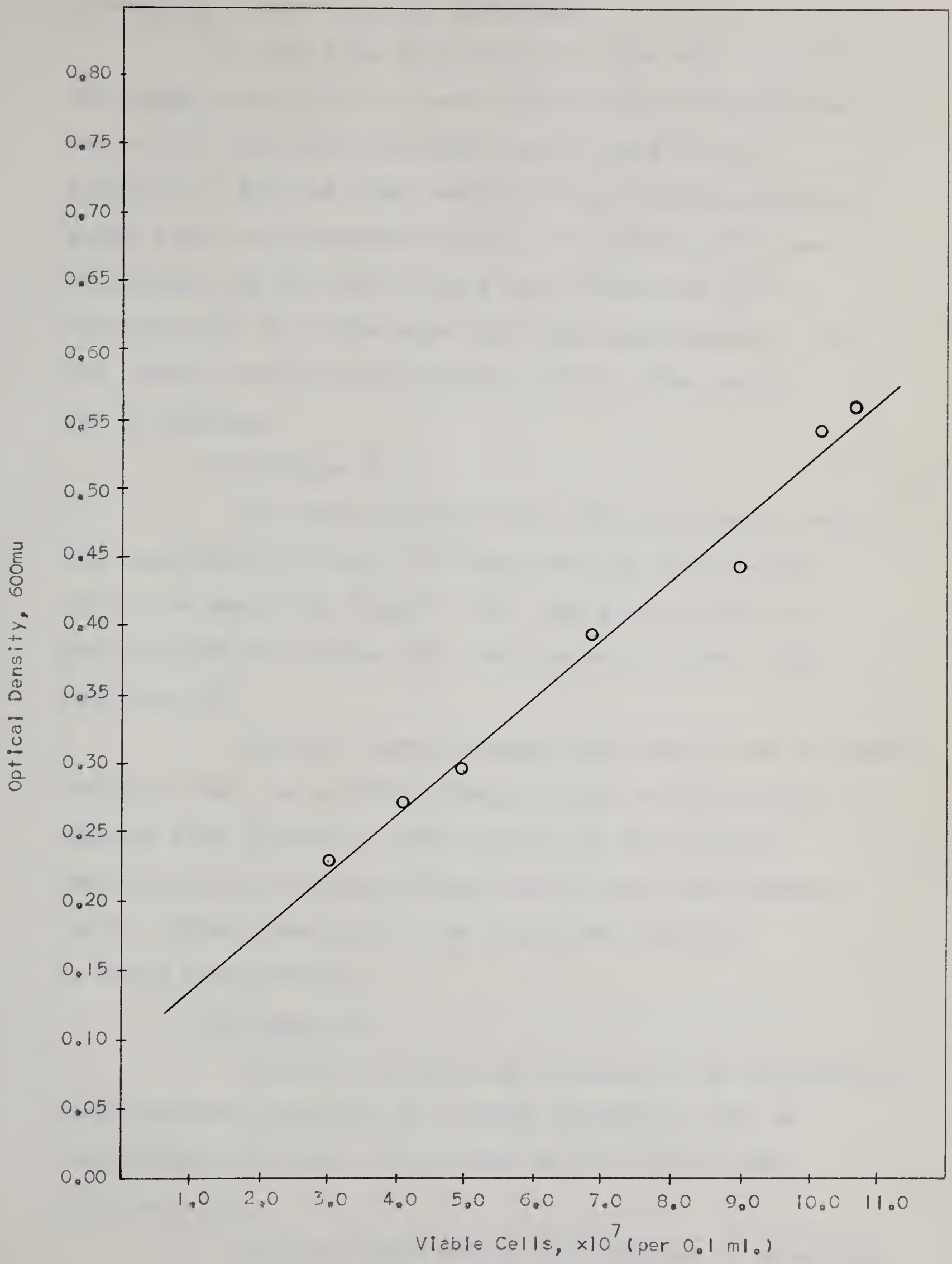

Pseudomonas fluorescens Growth Curve
Optical Density, 600 mu
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1.0
2.0
3.0
4.0
Hours of Incubation
Optical Density
Viable Cells
10^7
2
3
4
5
6
7 X 10^7
Log_{10} Viable Cells (per 0.1 ml.)

Figure 2

Pseudomonas fluorescens Growth Curve



determined at zero time by titration.

At zero time the bacterial titre was 2.5 X 10^7, the phage titre for the lower multiplicity of infection was 4 X 10^7 and for the higher multipliciy 4 X 10^8 (figure 3). For the lower multiplicity of infection the burst size was approximately 35, the latent period was 45 minutes and the rise period was 60 minutes. For a multiplicity of 10 the burst size was approximately 100, the latent period was 60 minutes and the rise period was 30 minutes.

(11) Phage B.

The same procedure was used for phage B with the exception that only a multiplicity of 10 was used. It can be seen from figure 5 that the latent and rise periods were 60 minutes each and the burst size of the phage was 50.

Neither phage produced clearing of the bacterial culture. When the optical density at 600 mu was plotted against time (figure 4) the increase in the optical density of the infected culture paralleled the increase in the optical density of the uninfected culture.

4. Phage Purification.

(1) Phage 16

Initial purification of phage 16 by differential centrifugation resulted in a phage suspension with an O.D.230/O.D.260 ratio of 1.00 and an O.D.260/O.D.280 ratio of 1.65.

Further purification was obtained by adsorbing

Figure 3
Phage 16 One Step Growth Curve

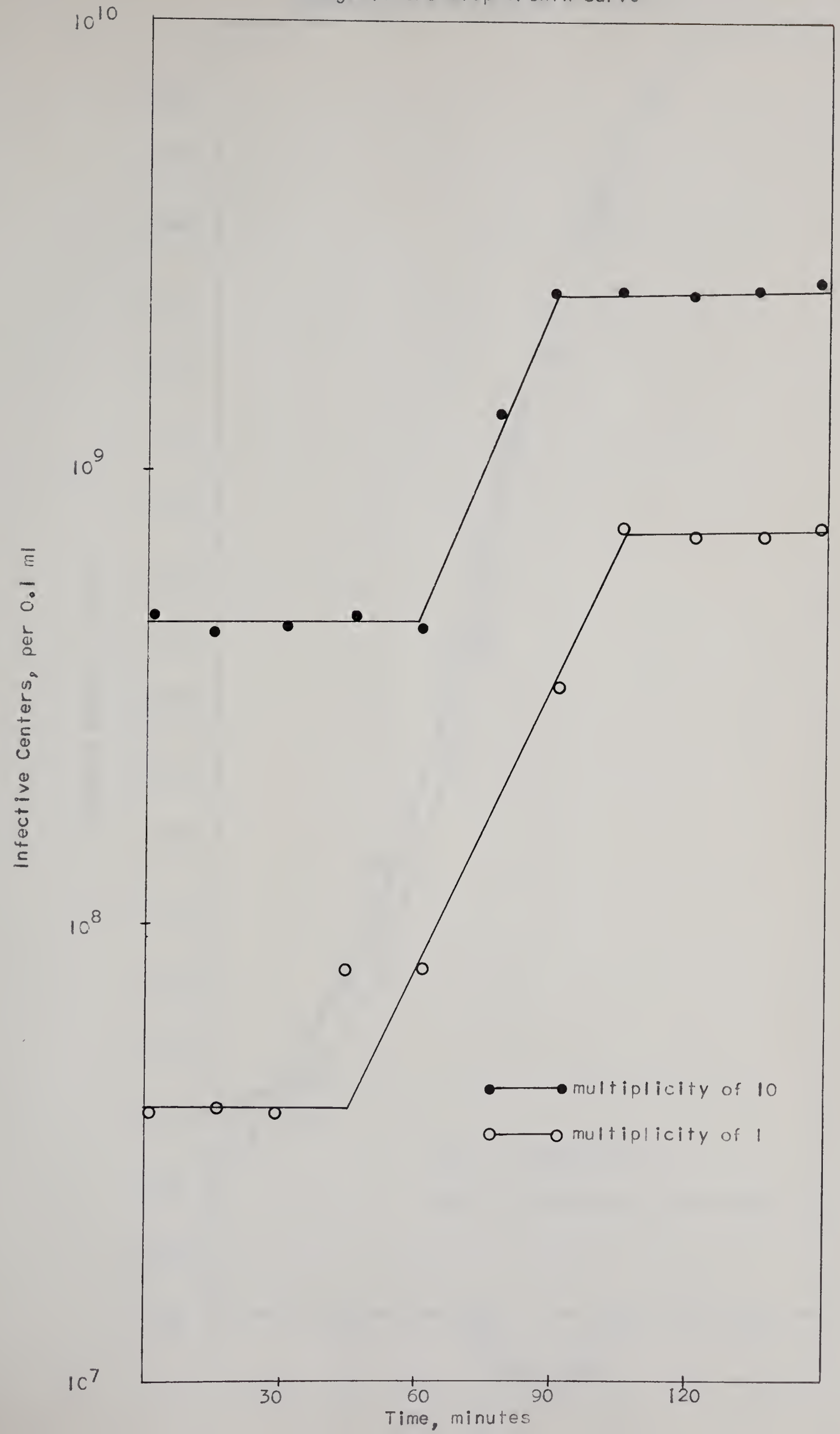


Figure 4
Pseudomonas fluorescens Growth Curve

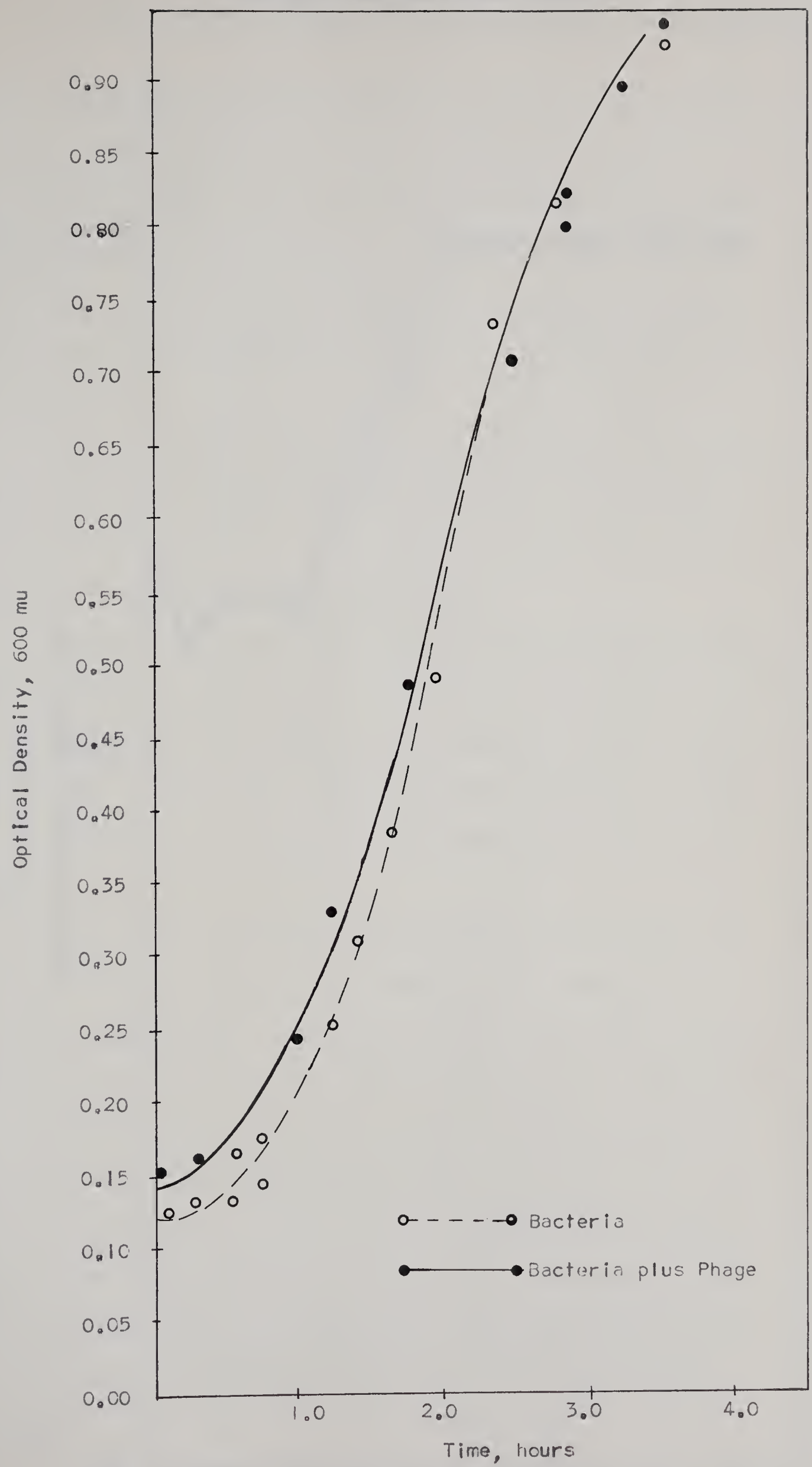
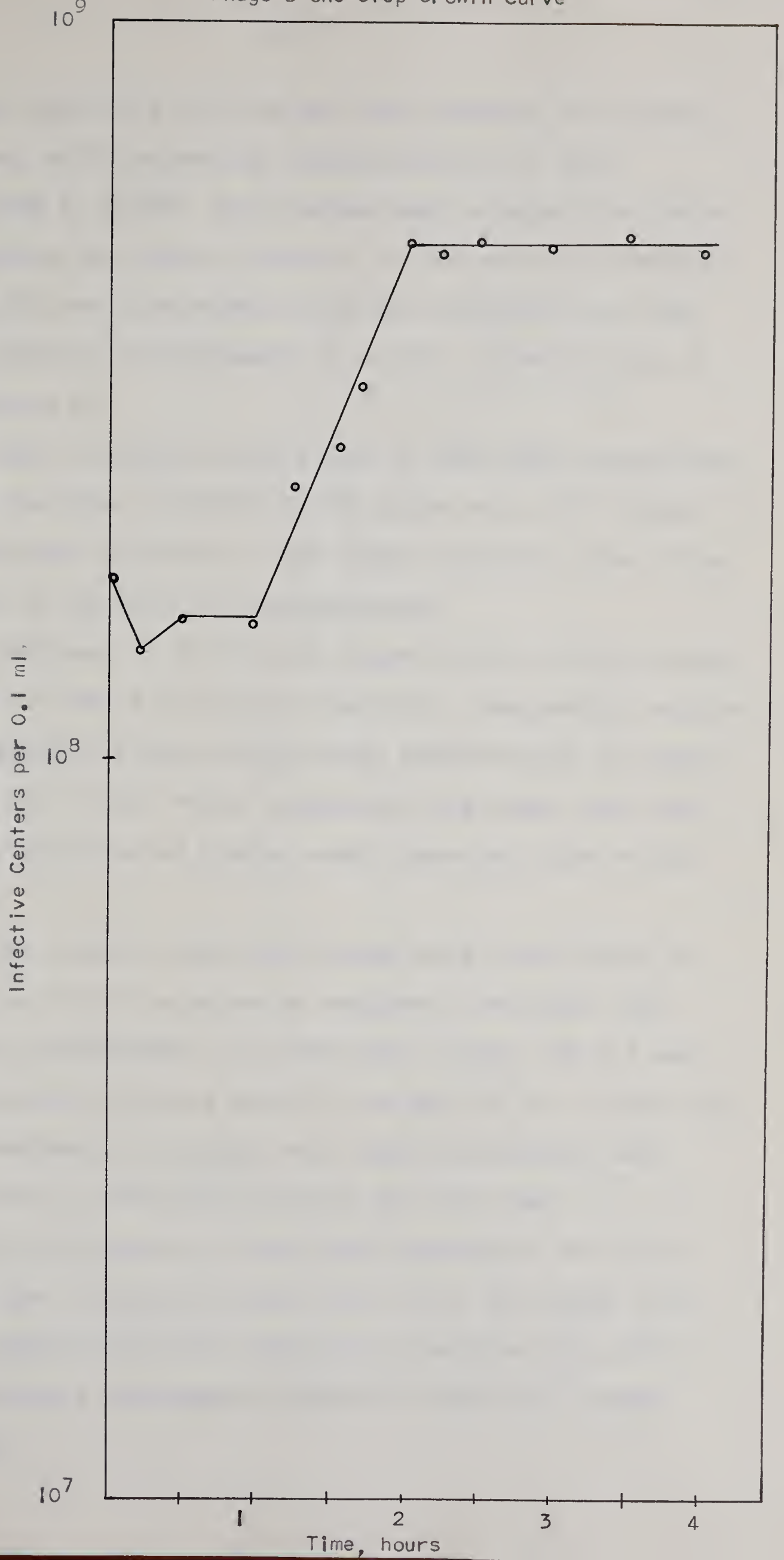


Figure 5

Phage B One Step Growth Curve



the phage on DEAE cellulose and then washing the column, batchwise, with increasing concentrations of NaCl (from 0.05M to 0.5M). The eluates were assayed for phage by measuring the optical density at 260 mμ and titration of the different fractions. Both the maximum titre and optical density corresponded to a NaCl concentration of 0.2M (figure 6).

The O.D.230/O.D.260 ratio of the phage suspension was 0.85 and the O.D.260/O.D.280 ratio was 1.50 (figure 7). The absorption spectrum of the phage solution from 200 mμ to 310 mμ is typical of nucleoprotein.

Recovery of infectious phage in the eluate ranged from 40% to 90% of the input (table 5). Comparable results were obtained for both large scale purification of phage, where 50 ml. of the virus suspension was used, and small scale purification of phage, where one-tenth the volume was used.

The eluates from the column were centrifuged at 24,000 rpm. for 30 minutes to sediment the phage. The pellet was resuspended in 0.04M Tris buffer, pH 7.3 and the preparation treated with RN'ase and DN'ase. After the enzyme treatment the phage were again sedimented and resuspended in 0.04M Tris buffer, pH 7.0. The O.D.230/O.D.260 ratio of the phage suspension was still 0.85 and the O.D.260/O.D.280 ratio 1.52. The phage titre was not affected and the absorption spectrum from 200 to 300 mμ remained unchanged (figure 7) after the enzyme treatment.

Figure 6

Column Chromatography of Phage 16

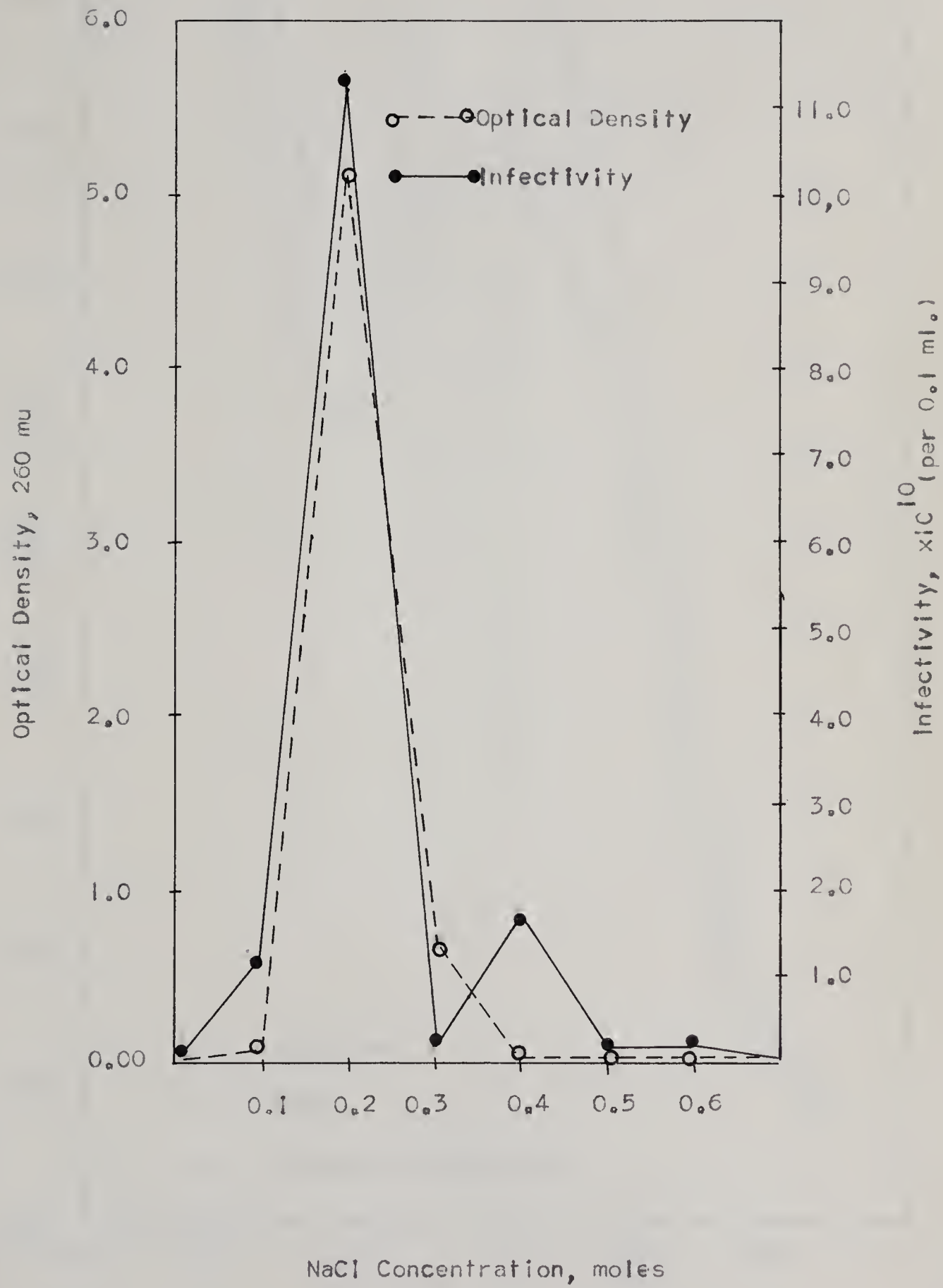


Figure 7

Ultraviolet Absorption Spectrum

of Purified Phage 16 Preperations

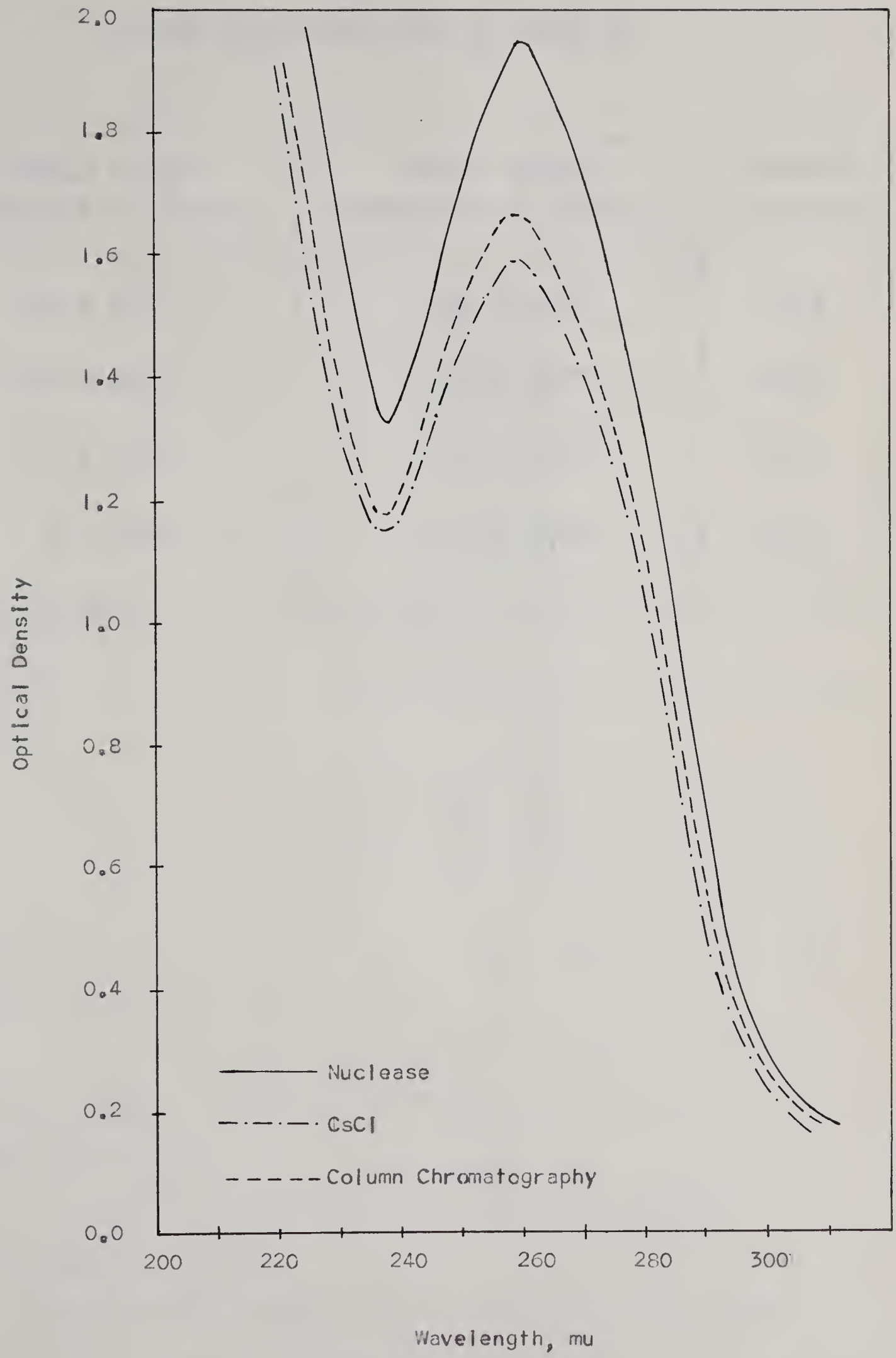


TABLE 5.

Column Chromatography of Phage 16

Total Phage Adsorbed in 50 ml.	Total Phage Recovered in 100 ml.	Percent Recovery
2.2×10^{13}	1.08×10^{13}	45.5
3.5×10^{14}	1.4×10^{14}	40.0
1×10^{13}	9.5×10^{12}	95.0
1×10^{13}	5.1×10^{12}	51.0

After enzyme treatment 0.8 ml. of the phage suspension (titre 4.8 X 10^{12}/0.1 ml.) was placed on a preformed CsCl gradient (1.23 gm.cm.$^{-3}$ to 1.63 gm.cm.$^{-3}$) and centrifuged for 45 minutes. The rotor head was allowed to come to a stop unbraked, and the tubes were fixed in a vertical position. The phage appeared as a distinct band near the centre of the centrifuge tube (plate 3). Four drop fractions were collected from the bottom of the tube after pinhole perforation. Both the maximum phage titre and optical density at 260 mμ corresponded to a density of 1.50 gm.cm.$^{-3}$. (figure 8).

The absorption spectrum of the phage suspension was typical for nucleoprotein (figure 7). The O.D.230/O.D.260 ratio was 0.83 and the O.D.260/O.D.280 ratio was 1.51. After density gradient centrifugation 91% of the infectious phage were recovered.

The particle weight of phage 16 can be calculated from the diameter and density of the phage (Adams, 1959). Once the particle weight of the phage is known, one can calculate the molecular weight of the phage. However, the value will not be accurate since

(a) the phage diameter will be smaller due to shrinkage after treatment for electron microscope observation, and

(b) the density will be high due to hydration of the phage particle.

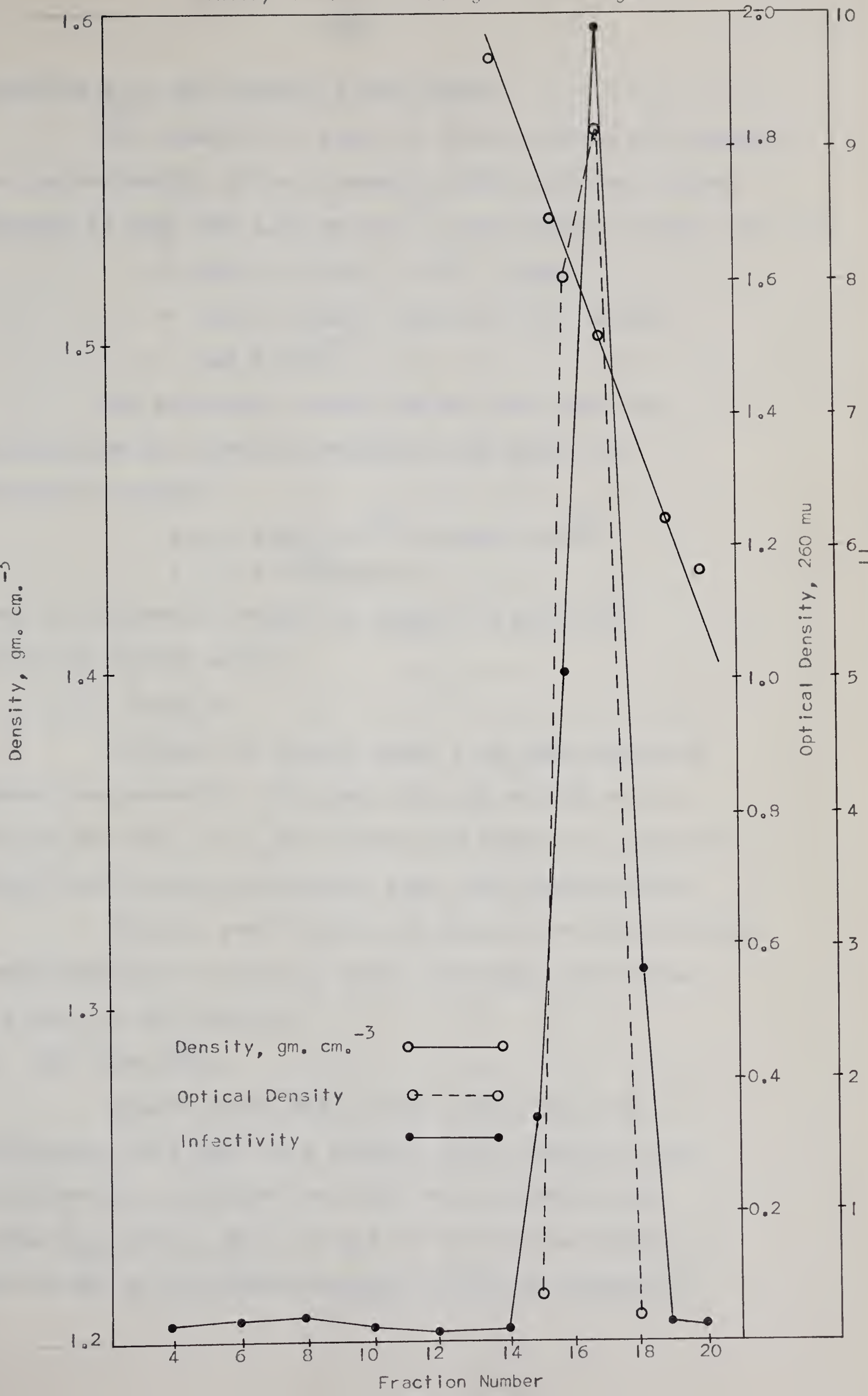
The particle weight will be given by the formula $4/3 \quad R^3$ X Density (Adams, 1959, page 46). In the

PLATE 3.

Phage 16 in CsCl Density Gradient

Figure 8

Density Gradient Centrifugation of Phage 16



equation R is the radius of the phage.

The diameter of phage 16 from electron micrographs was approximately 50 mu (Yamamoto, 1964) and the buoyant density in CsCl was 1.50 gm.cm.$^{-3}$. The particle weight will be;

$$= (4/3)\ (3.14)\ (25)^3\ (1.50)$$
$$= (4/3)\ (3.14)\ (25 \times 10^{-7})^3\ (1.50)$$
$$= 9.8 \times 10^{-17}$$

The molecular weight can be calculated by multiplying the particle weight of the phage by Avagadro's number,

$$\text{i.e. } 9.8 \times 10^{-17} \times 6.023 \times 10^{23}$$
$$= 59 \times 10^{6} \text{daltons}$$

Thus the molecular weight of phage 16 is 6×10^{7} molecular weight units.

(11) Phage B

Attempts to purify phage B on DEAE cellulose proved unsuccessful. The phage did not adsorb on the column and only 1% of the infectious phage was recovered. Other purification procedures were also unsuccessful.

Partial purification of phage B by differential centrifugation is possible since the phage titre does not drop in the process.

5. DNA Extraction

Salmon sperm DNA (Grade A obtained from Calbiochem) was used as a control in all nucleic acid experiments. A standard solution was prepared using 0.04M PO_4 buffer, pH 7. It had an absorption minimum at 230 mu and an absorption maximum at 259 mu (figure 9).

The O.D.230/O.D.260 ratio was 0.43 and the O.D.260/O.D.280 ratio was 2.00.

Nucleic acid isolated from phage 16 and P. fluorescens by treatment with phenol, had an absorption spectrum similar to that of the salmon sperm DNA (figure 9). The preparations gave a positive diphenylamine reaction indicating that the nucleic acid was DNA (Disch, 1955). The minimum, maximum and optical density ratios of the DNA's are compared in table 6.

Removal of phenol from the DNA preparation was assayed by the O.D.260/O.D.280 ratio. (Phenol absorbs strongly at 270 mμ). Removal of protein was assayed by the absorption minimum wavelength. If protein is present, the minimum will move from a wavelength of approximately 230 mμ characteristic for DNA to a higher wavelength.

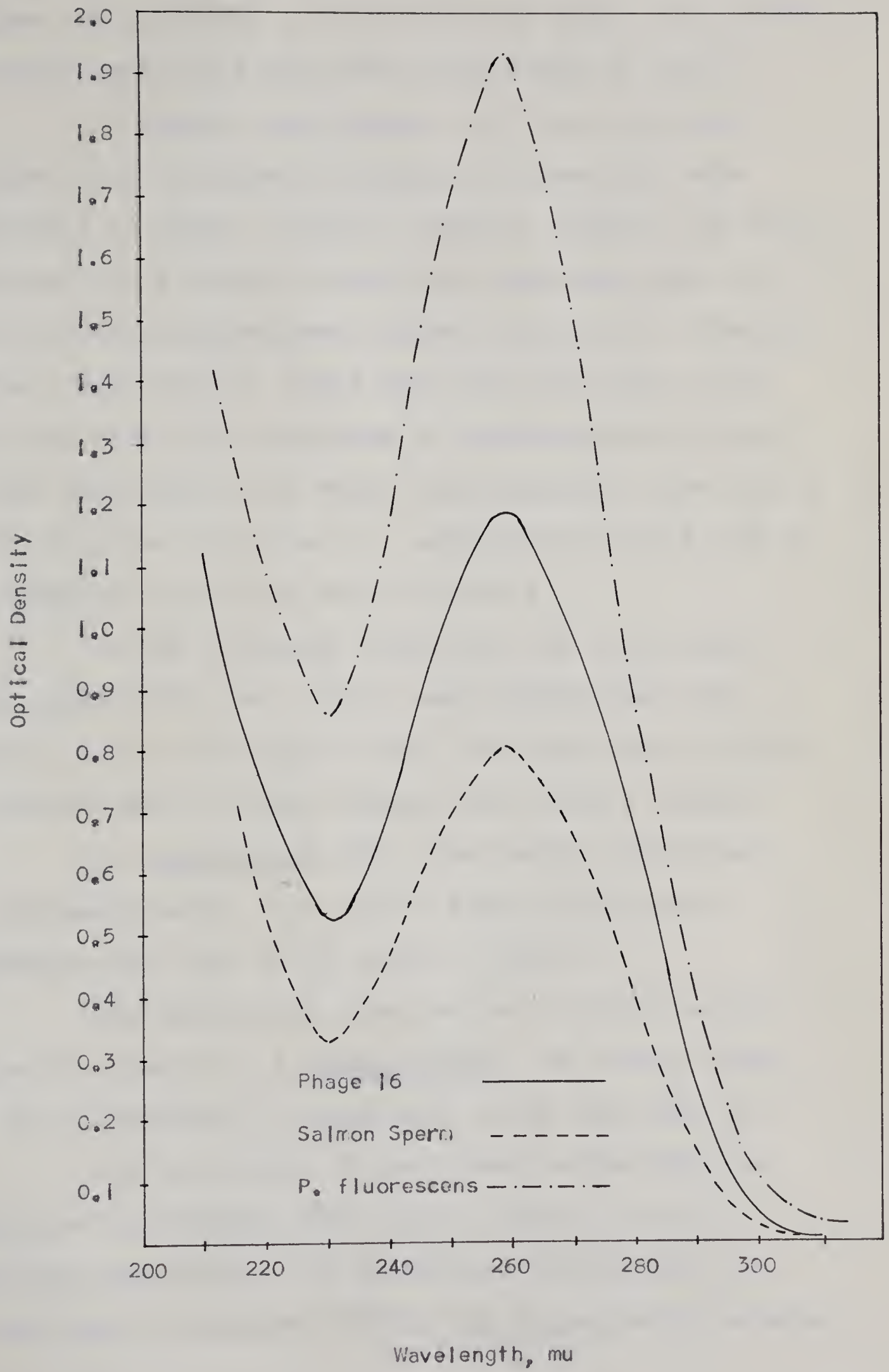
DNA at a concentration of approximately 1.0 mg. per ml. was obtained by phenol extraction. For example, it was possible to obtain a 3 ml. volume of nucleic acid from 5 ml. of a phage suspension containing 1×10^{13} phage per ml.. At 260 mμ a 1/15 dilution of this nucleic acid preparation had an optical density of 1.5. This meant that the original solution contained $3 \times 15 \times 1.5 = 67.5$ O.D. units of DNA. If approximately 20 O.D. units are equivalent to 1 mg. of DNA, then in 3 mls. there would be 3.5 mg. of DNA or approximately 1.1 mg of DNA per ml..

6. Chemical Analysis of DNA

Hydrolysis of phage 16 DNA, according to the

Figure 9

Ultraviolet Absorption Spectrum of DNA's



method of Wyatt (1955), yielded four spots with Rf values corresponding to adenine, guanine, cytosine and thymine. In addition, a fifth but very faint ultraviolet absorbing spot was found with an Rf value of uracil.

In control experiments the bases (A grade obtained from California Biochemical Research) were hydrolysed as above. Adenine, guanine, thymine and uracil each gave one distinct ultraviolet absorbing spot with Rf values of the unhydrolysed controls (plate 4). Cytosine, however, was found to yield two spots. One had an Rf value and absorption spectrum of unhydrolysed cytosine and the other had an Rf value and absorption spectrum of uracil (figure 10; plate 4). Approximately 1% to 5% of the cytosine was broken down to uracil.

If the cytosine, which did not break down during hydrolysis, was eluted and rehydrolysed two separate spots were again found. The breakdown product, if rehydrolysed, did not undergo any further change.

P. fluorescens DNA, after being hydrolysed and chromatogramed, also gave a faint ultraviolet absorbing spot with an Rf value of uracil.

The absorption spectrum and optical density ratios for phage 16, P. fluorescens, and salmon sperm DNA are illustrated in figures 11 to 13 and table 7.

The percentage of each base in the DNA was calculated by dividing the optical density at the absorption maximum by the extinction coefficient (ε) as published by Bendich (1957). The relationship between

Figure 10

Ultraviolet Absorption Spectrum

of Uracil and Hydrolysed Cytosine

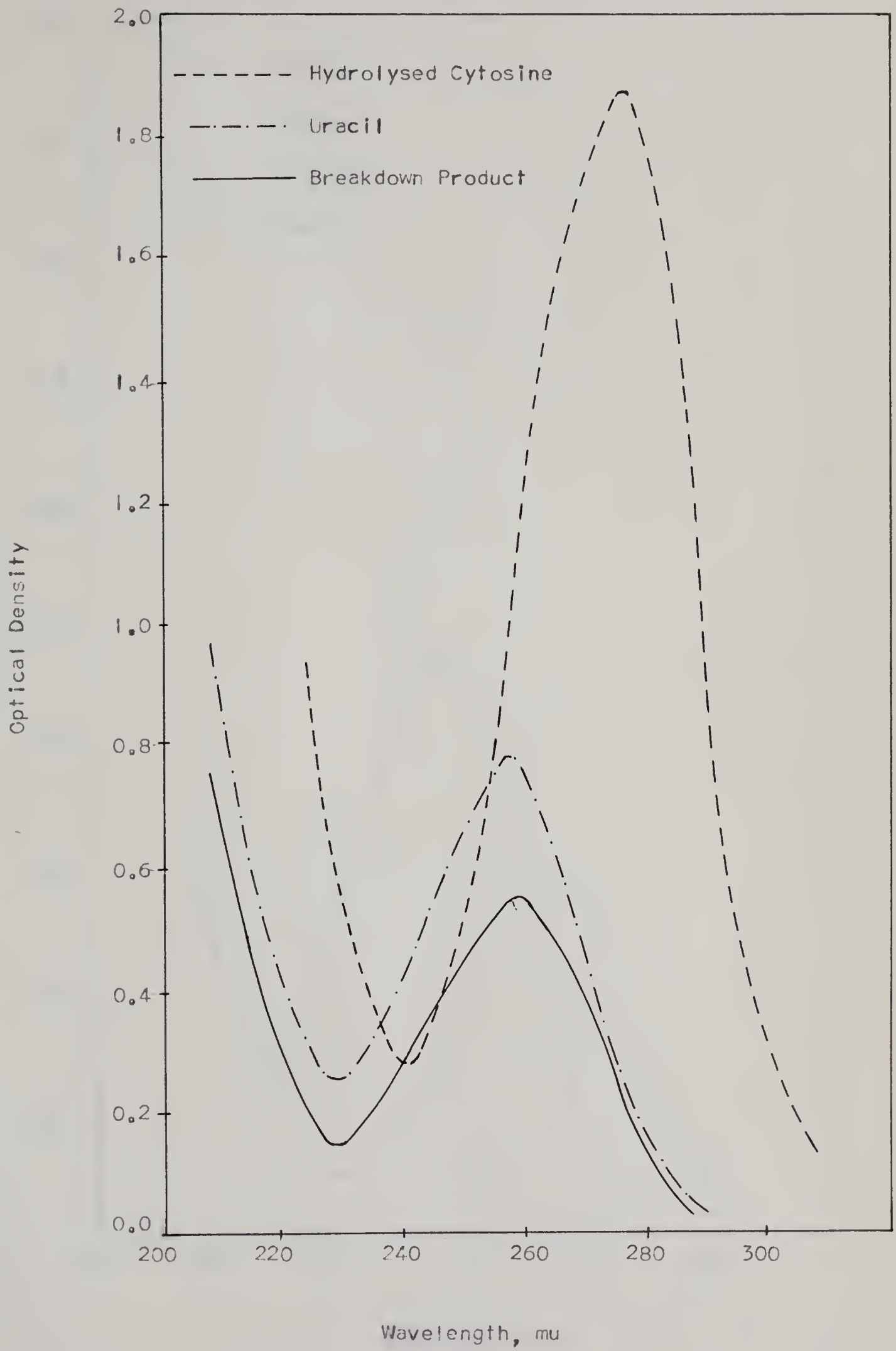


Figure 11

Ultraviolet Absorption Spectrum

of Salmon Sperm DNA Hydrolysates

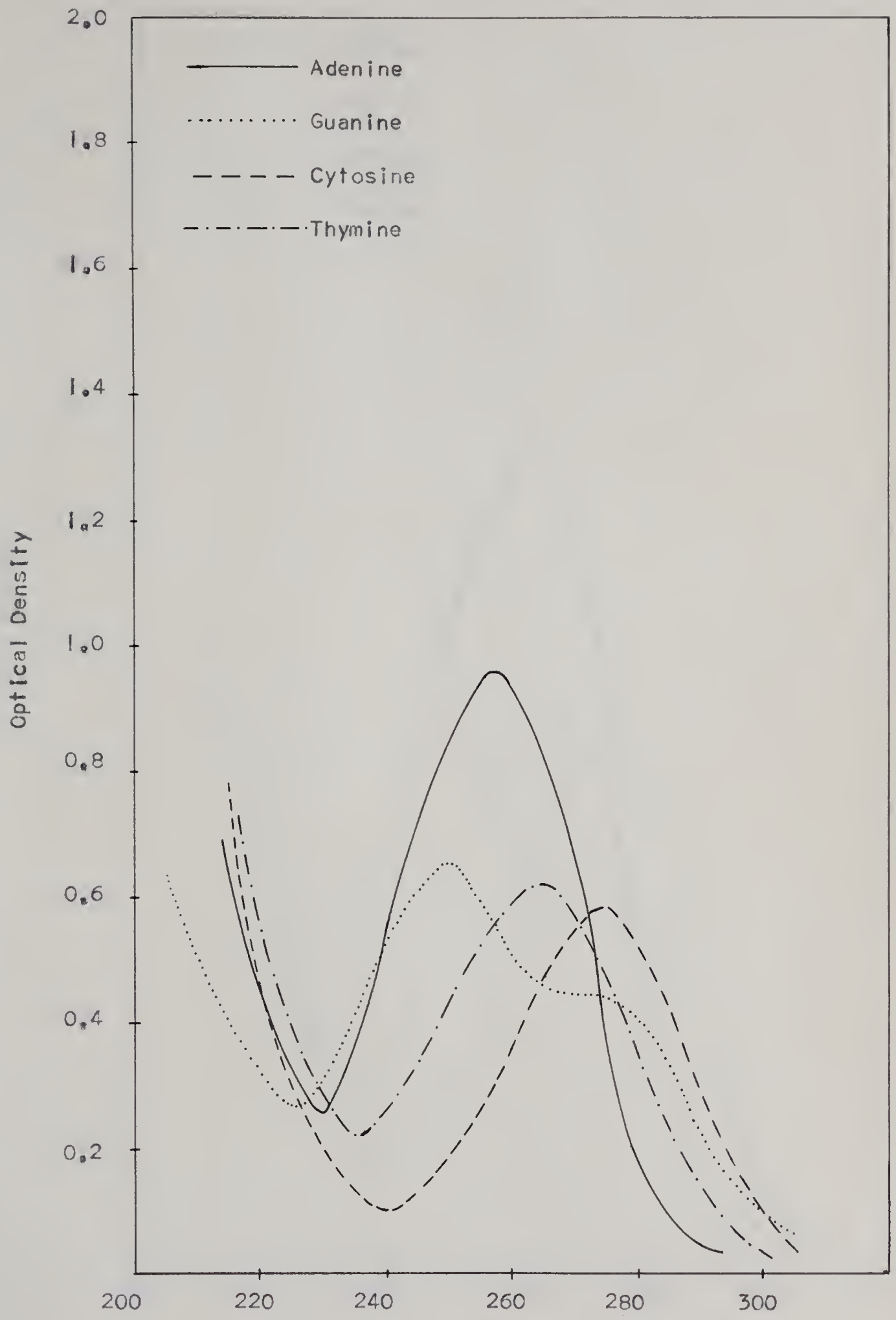
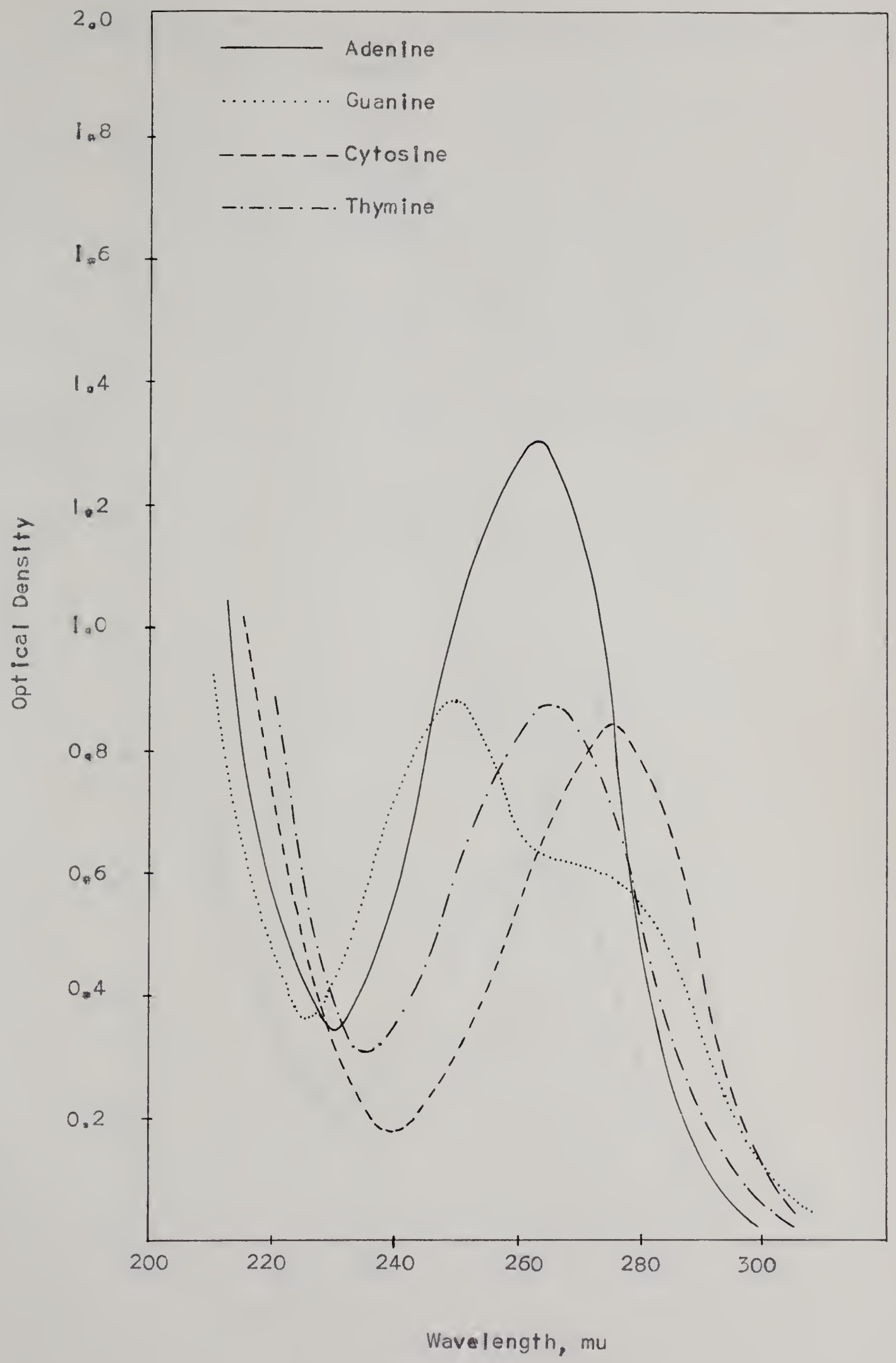


Figure 12

Ultraviolet Absorption Spectrum

of Phage 16 DNA Hydrolysates



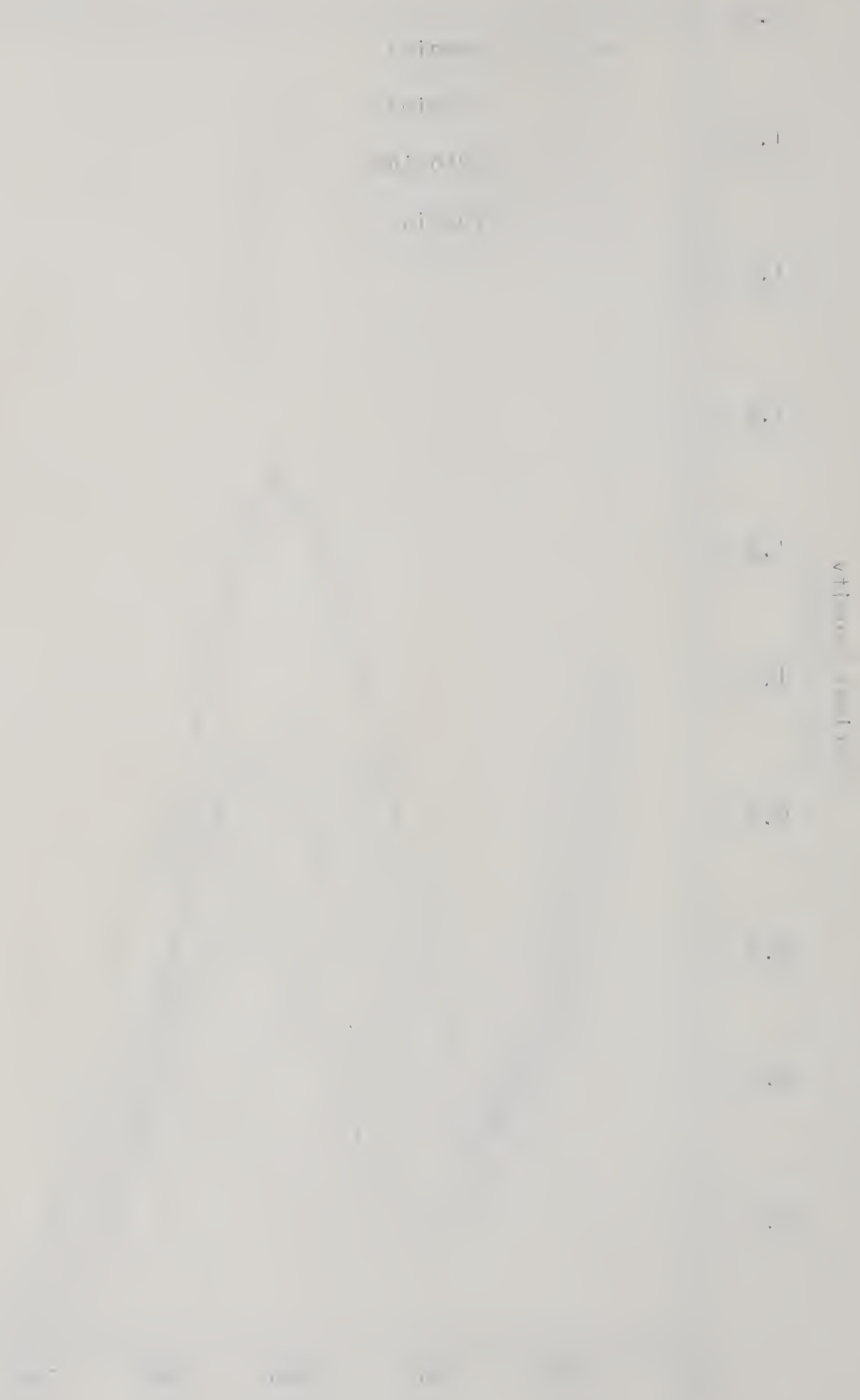


Figure 13

Ultraviolet Absorption Spectrum of

Pseudomonas fluorescens DNA Hydrolysates

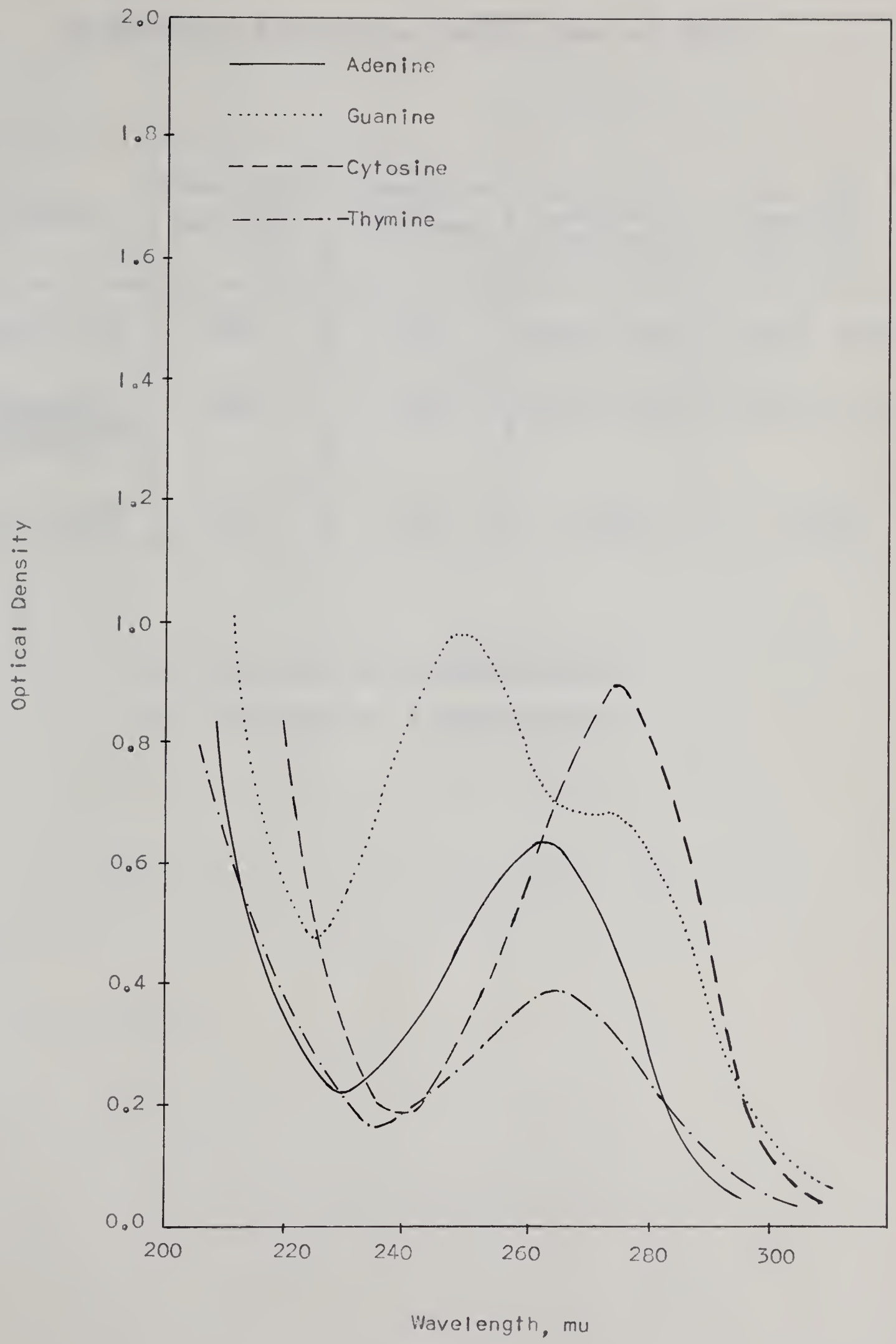


TABLE 6.

Ultraviolet Absorption Spectra Data of DNA's

DNA Source	Absorption Maximum	Absorption Minimum	$\frac{\text{O.D. 230}}{\text{O.D. 260}}$	$\frac{\text{O.D. 260}}{\text{O.D. 280}}$
Phage 16 (1)	260	231	0.45 ± 0.02	1.93 ± 0.02
Pseudomonas fluorescens(2)	260	231	0.45 ± 0.02	1.93 ± 0.02
Salmon Sperm	259	230	0.43	2.00

(1) Average of 10 experiments.

(2) Average of 5 experiments.

TABLE 7.

Ultraviolet Absorption Spectra Data of Bases

Base	O.D. 250 / O.D. 260	O.D. 280 / O.D. 260	Source of Bases (chromatogramed and eluted in N/10 HCl)
A	0.81	0.39	unhydrolyzed control bases (California Biochemical Research, A Grade).
G	1.33	0.78	
T	0.72	0.57	
C	0.48	1.49	
A	0.80	0.39	hydrolysed control bases (California Biochemical Research, A Grade).
G	1.30	0.81	
T	0.71	0.51	
C	0.54	1.59	
A	0.79	0.45	salmon sperm DNA
G	1.28	0.79	
T	0.71	0.64	
C	0.51	1.54	
A	0.80	0.41	phage 16 DNA
G	1.25	0.79	
T	0.75	0.63	
C	0.50	1.56	
A	0.79	0.48	P. fluorescens DNA
G	1.25	0.79	
T	0.69	0.64	
C	0.52	1.48	
C	0.51	1.52	hydrolysed cytosine
X*	0.78	0.26	cytosine breakdown product
U	0.78	0.19	uracil

X* = unknown

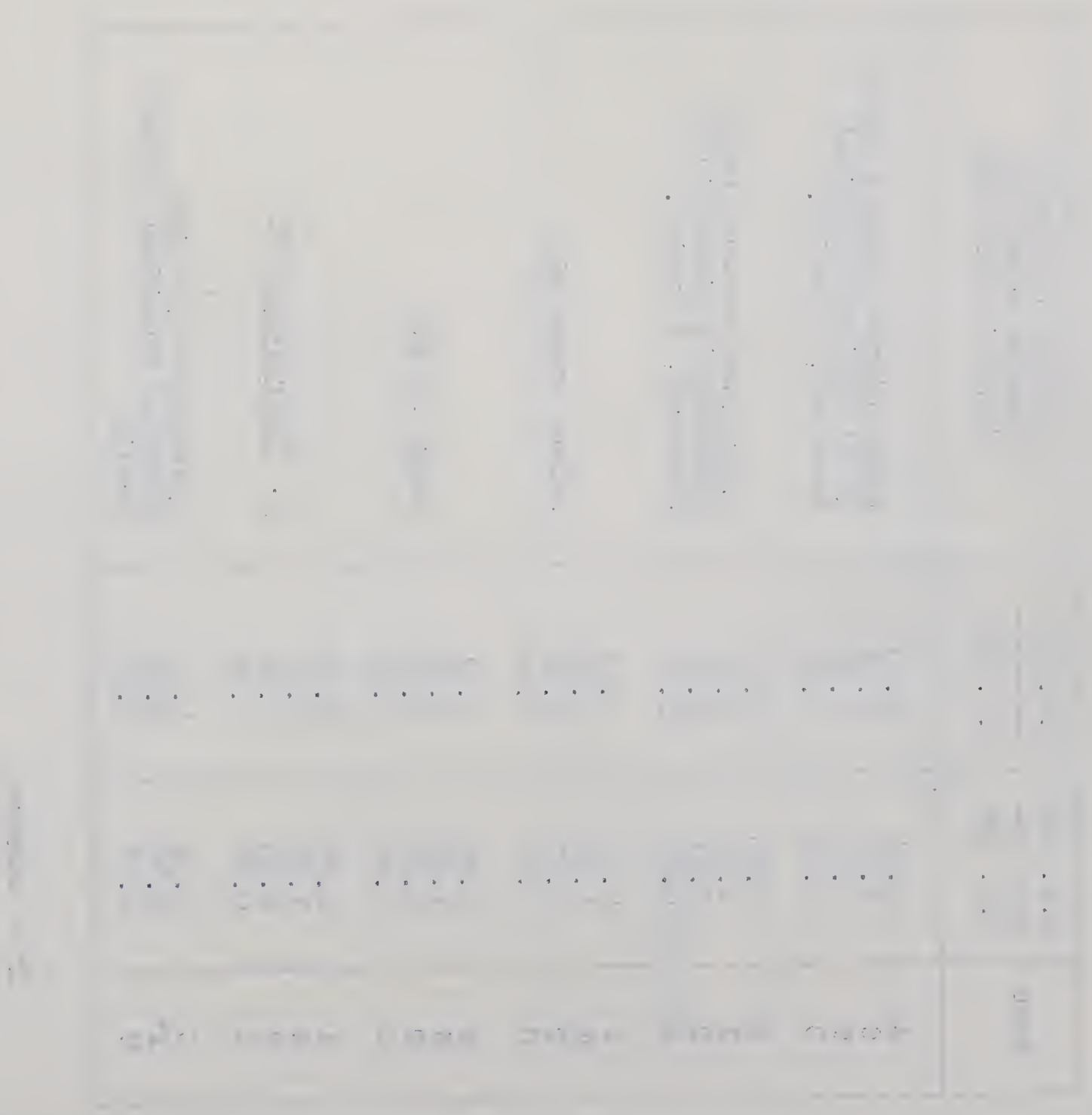
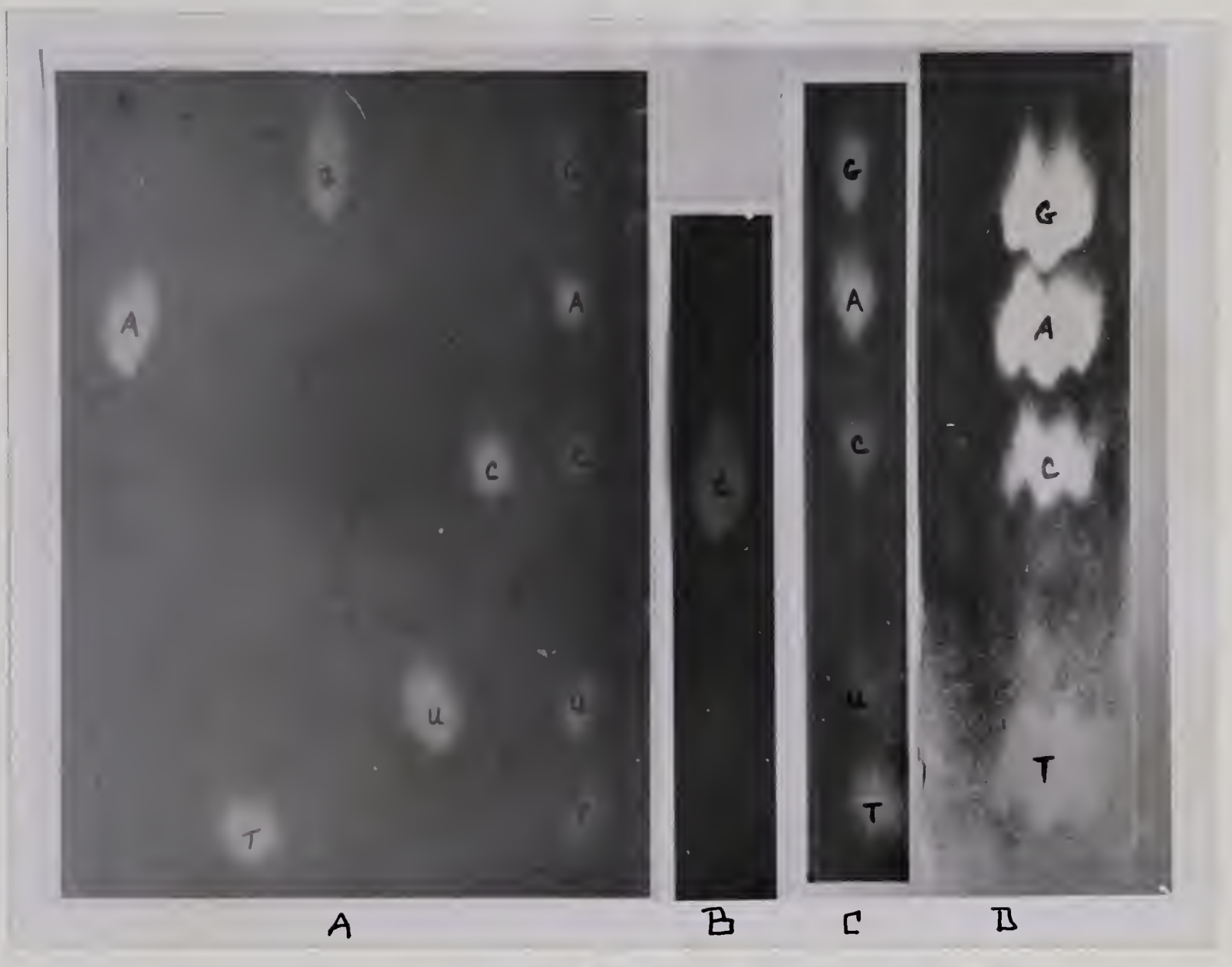


PLATE 4.

Hydrolysates of Phage 16 and Salmon Sperm DNA



A. Unhydrolysed Control Bases

B. Hydrolysed Cytosine

C. Hydrolysed Phage 16 DNA

D. Hydrolysed Salmon Sperm DNA

the extinction coefficient and optical density is given by the equation:

$$\text{molarity} = {}^{O.D.}/_{\varepsilon} \quad \text{Bendich (1957)}$$

An example of the determination of the mole percent of each base for phage 16 follows:

Base	O.D.	÷	X 10^{-3}	=	molarity
A	0.94	÷	12.6	=	0.075
T	0.62	÷	7.95	=	0.078
G	0.615	÷	11.1	=	0.055
C	0.47	÷	10	= 0.047	0.056 *
U	0.07	÷	8.15	= 0.009	

* When some of the cytosine was degraded to uracil the amount of uracil was calculated and added on to the amount of cytosine in the preparation. This value should represent the total amount of cytosine originally in the DNA preparation. Table 8 lists the mole percent of each base found in P. fluorescens, phage 16 and salmon sperm DNA.

The percent recovery of the bases was calculated from the phosphorus content of the hydrolysate. The phosphorus, as phosphate, was determined by measuring the optical density, at 600 mμ, of a fixed volume of the hydrolysate and relating this by a standard curve to the mg. of phosphorus in the sample. The value was converted to moles of phosphorus by dividing the weight obtained from the standard curve by the molecular weight of phosphate i.e.

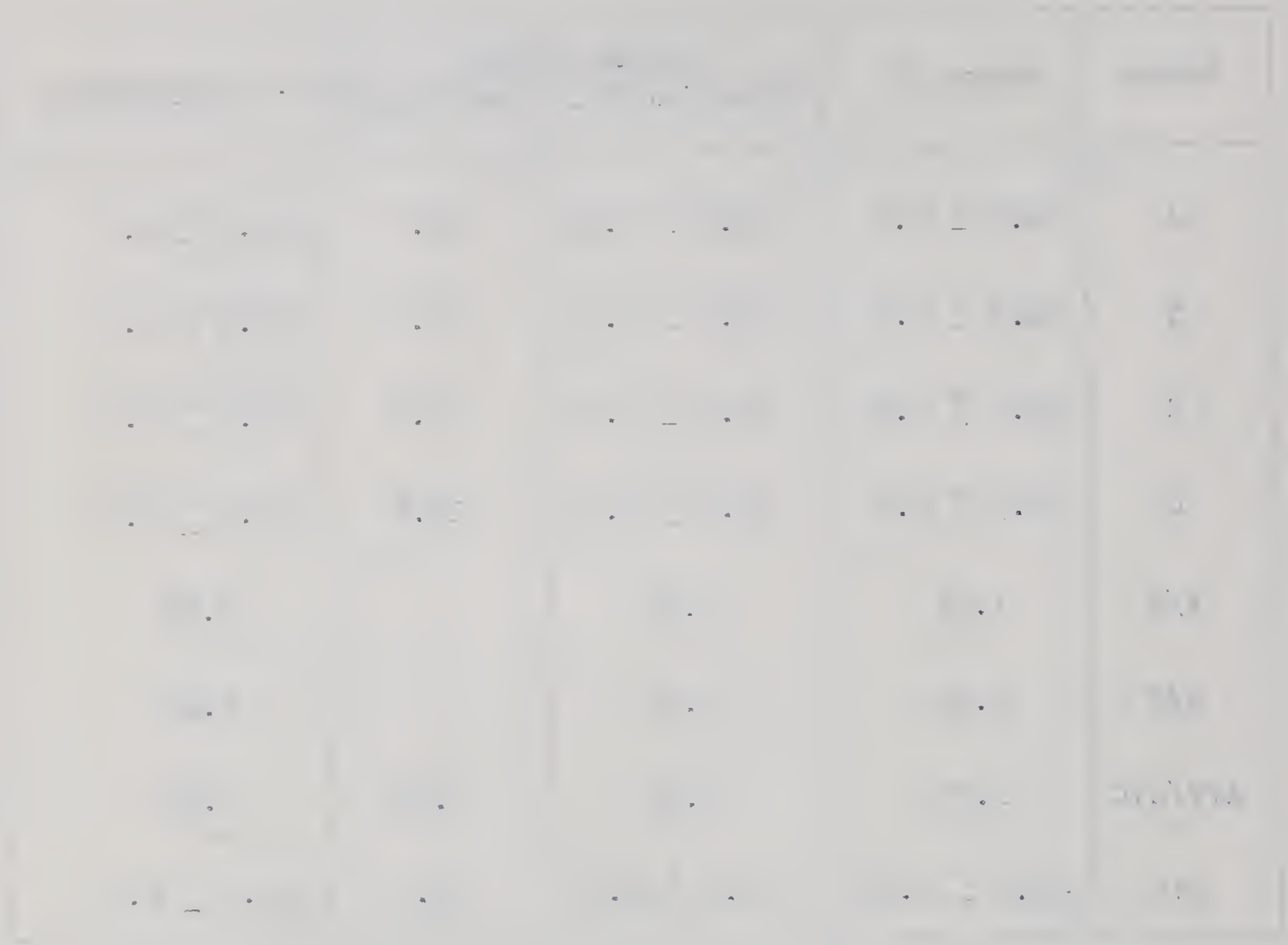
$$\text{moles (m)} = \text{weight (w)} / \text{molecular weight (M)}$$

TABLE 8.

Mole Percent of Bases

Bases	phage 16	Salmon Sperm		*P. fluorescens*
		experimental	published*	
A	28.7 ± 0.5	28.9 ± 0.6	29.7	17.5 ± 0.7
T	28.5 ± 0.7	29.0 ± 0.6	29.1	17.8 ± 0.8
G	21.3 ± 0.5	21.1 ± 0.4	20.8	32.6 ± 0.5
C	21.5 ± 0.5	21.0 ± 0.5	20.4	32.1 ± 0.7
A/T	1.01	0.99		0.98
G/C	0.99	1.00		1.02
A+T/G+C	1.35	1.38	1.43	0.56
G+C	42.8 ± 1.00	42.2 ± 0.9	41.2	64.7 ± 1.2

* Chargaff, E., Lipshitz, R., and Green, C., (1952).



The micromoles of each base was determined by multiplying the optical density of the solution at its maximum by the volume in ml. used to elute the base, and the micromole optical density unit as published by Bendich (1957). A sample calculation for salmon sperm DNA together with the values found for phage 16 and P. fluorescens DNA follow

Salmon Sperm DNA:

base	O.D.		ml.to elute		micromole O.D.unit	
A	1.25	X	5	X	0.0794	= 0.498
T	0.80	X	5	X	0.1260	= 0.505
G	0.85	X	5	X	0.0901	= 0.382
C	0.77	X	5	X	0.1000	= 0.385

total micromoles base = 1.77

Now 5 λ of the hydrolysate had an optical density of 0.08. From the standard curve this is found to be eqivalent to 0.22 mg. P/ml. In 40 λ there would be

0.22/5 X 40 = 0.176 mg. P/ml.

Moles P = w/M = .176/95 = 0.00185

micromoles of P = 0.00185 X 1000 = 1.85

The percent recovery = 1.77/1.85 X 100 = 94%

The percent recovery for phage 16 DNA and P. fluorescens DNA was found to be 92% and 91% respectively.

7. Density Gradient Centrifugation of DNA

After centrifugation, phage 16 DNA appeared as a distinct band in the centrifuge tube. The DNA, assayed by measuring the optical density at 260 mμ, was found in the fraction corresponding to a buoyant density in CsCl

of 1.705 gm.cm.$^{-3}$(figure 14).

Sueoka etal (1959) and Rolfe and Meselsohn (1959) showed that the buoyant density in CsCl of DNA from various samples was linearly related to the mole percent guanine plus cytosine. By using figures published in the literature (Ben-Porat and Kaplin, 1962; Schildkraut etal, 1962; Marmur etal, 1963; Erikson and Szybalski, 1964) a curve was constructed relating buoyant density in CsCl to the mole percent guanine plus cytosine (figure 16).

From the curve it can be seen that a buoyant density of 1.705 gm.cm.$^{-3}$would correspond to a guanine plus cytosine content of 44%.

P. fluorescens DNA centrifuged in a density gradient also appeared as a distinct band in the centrifuge tube. The buoyant density of the DNA assayed as before was found to be 1.725 gm.cm.$^{-3}$ (figure 15). This would be equivalent to a guanine plus cytosine content of 65% (figure 16).

The molecular weight of the DNA in phage 16 can be determined from the mg. of phosphorus in the hydrolysate. For example,in one experiment, there was 0.12 mg. P/ml. or 0.00012 g.P/ml.. The phage titre was 2×10^{13}/ml. which means that there will be

$$\frac{1.2 \times 10^{-4}}{2 \times 10^{13}} = 0.6 \times 10^{-17} \text{g. DNA P/phage particle}$$

or

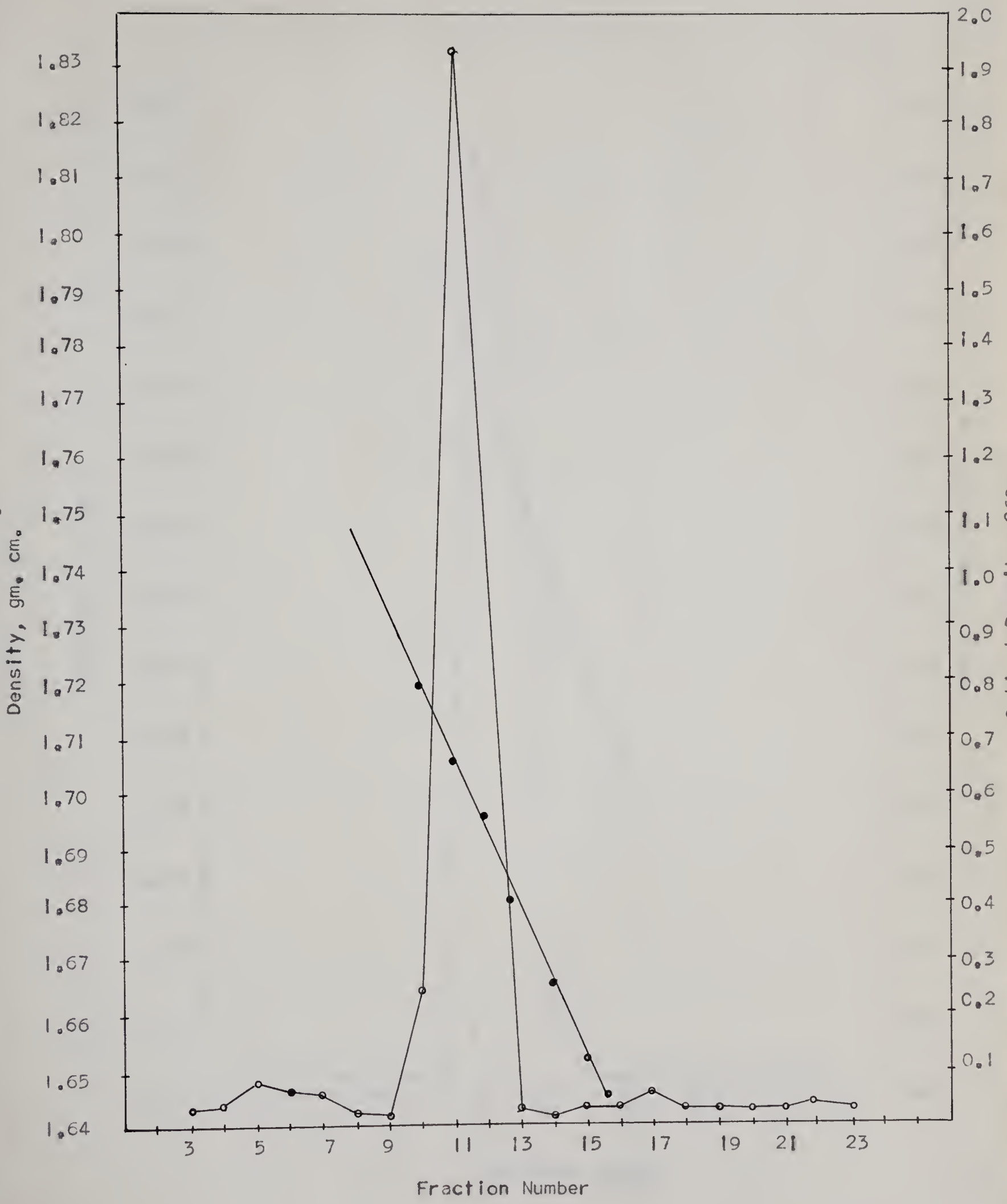
$$0.6 \times 10^{-16} \text{g. DNA / phage particle}$$

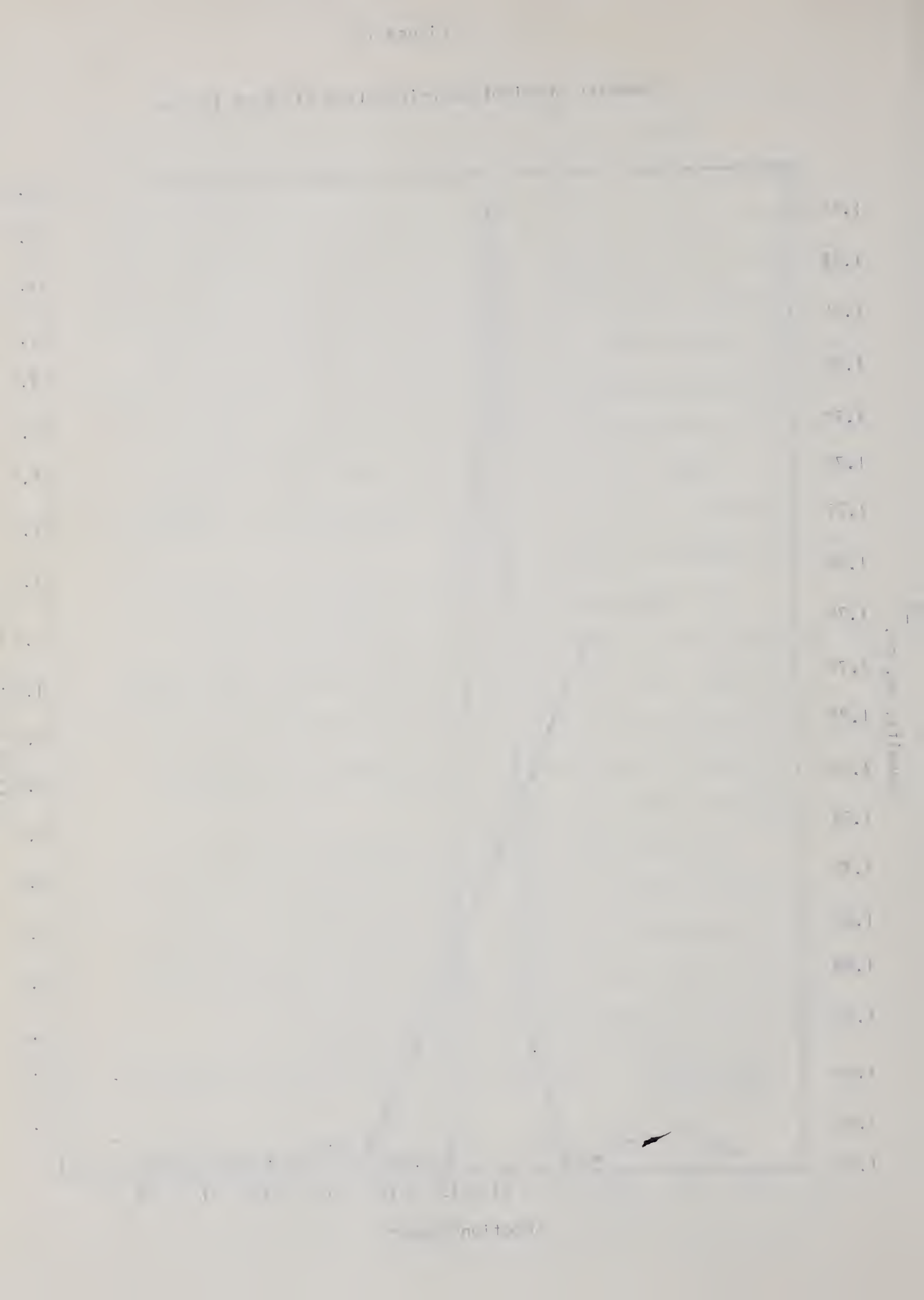
This would correspond to a molecular weight of

$$0.6 \times 10^{-16} \times 6.023 \times 10^{23} = 36 \times 10^{6} \text{ daltons.}$$

Figure 14

Density Gradient Centrifugation of Phage 16 DNA





Figure, 15

Density Gradient Centrifugation of P. fluorescens DNA

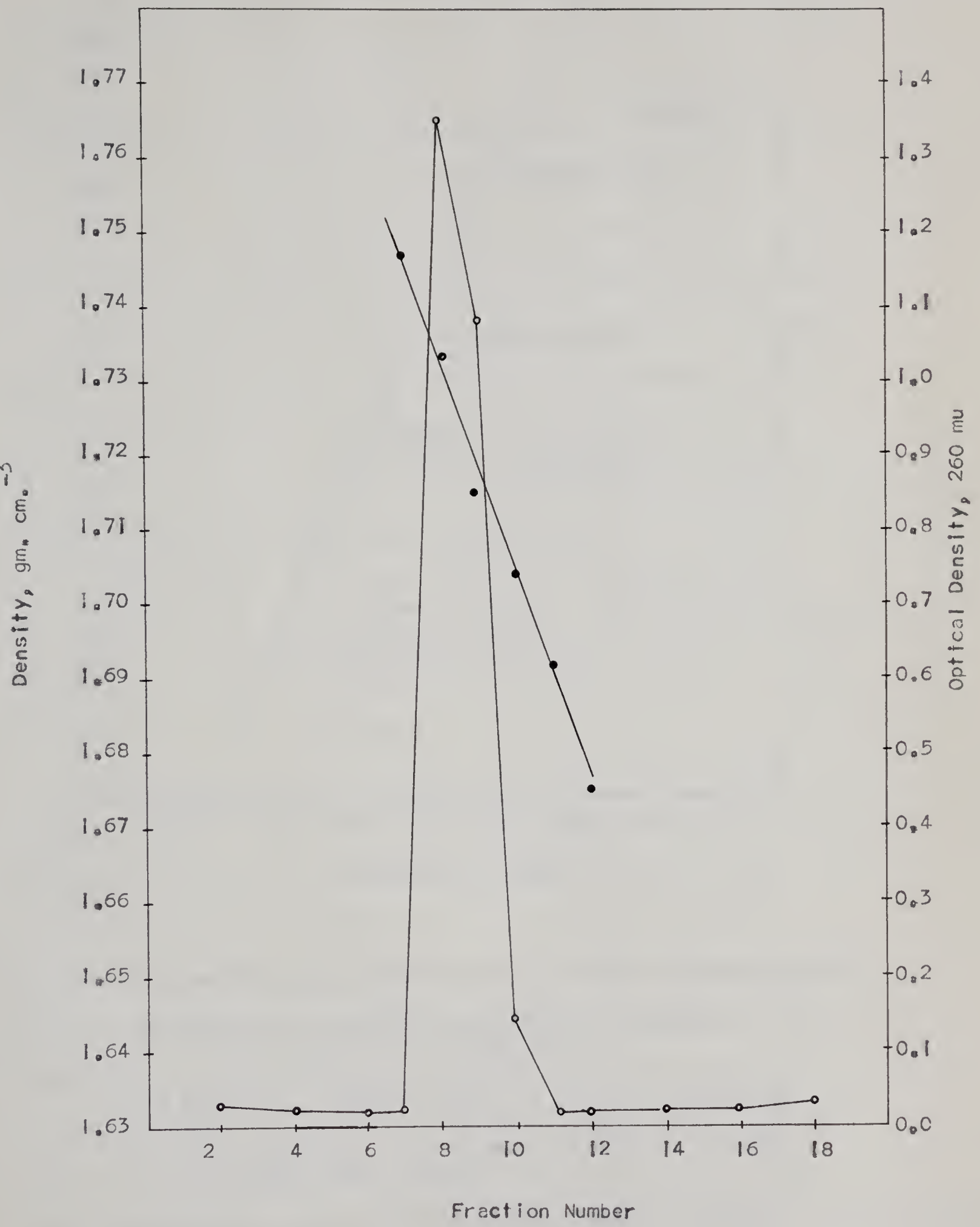
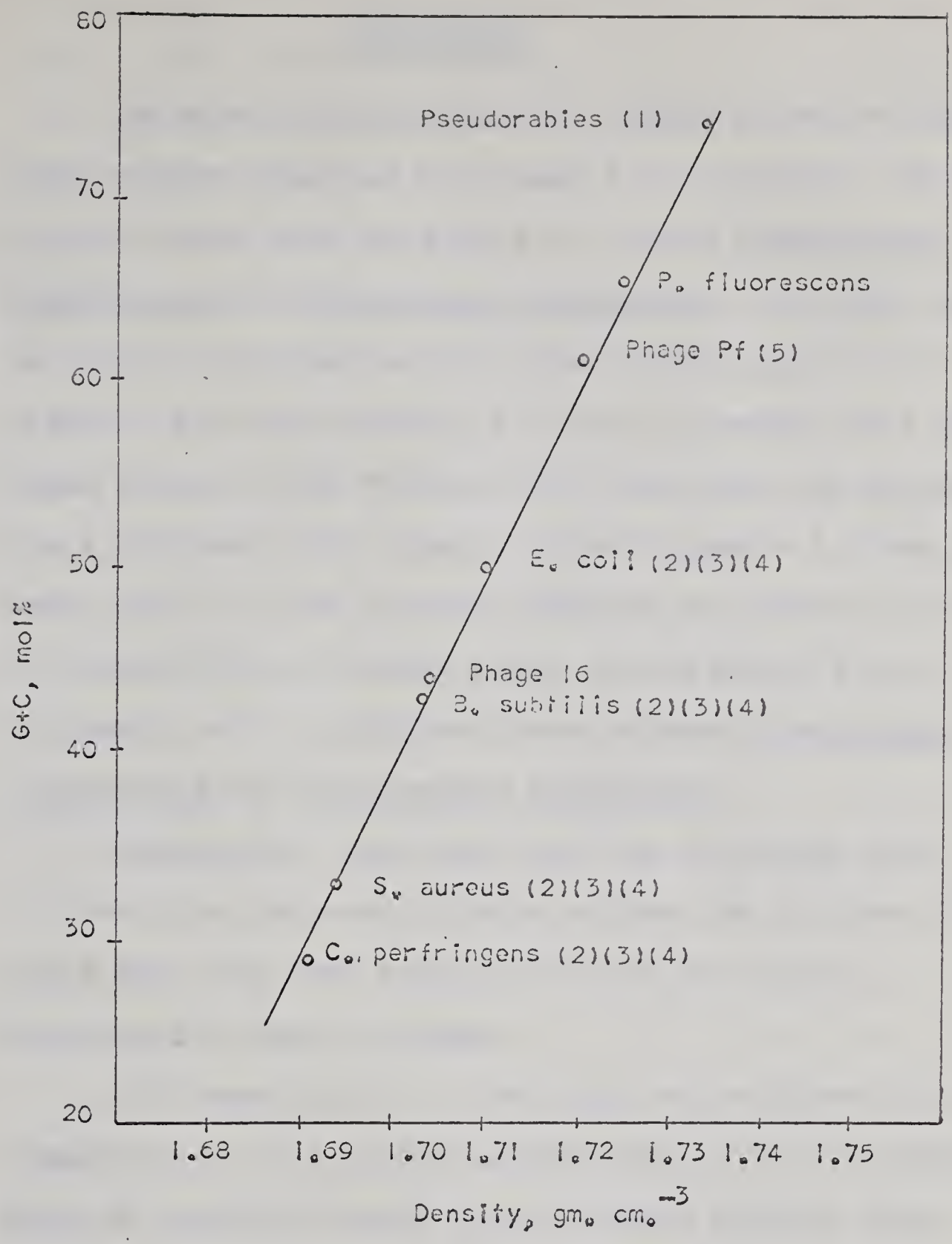


Figure 16



(1) Ben-Porat,T., and Kaplan,A.S., (1962). Virology 16, 261.

(2) Marmur,J., Falkow,S., and Mandel,M., (1963). Ann. Rev. Microbiol. 16, 329.

(3) Erikson,L., and Szybalski,W., (1964). Virology 22, 111.

(4) Schildkraut,C.L., Marmur,J., and Doty,P., (1964). J. Mol. Biol. 4, 430.

(5) Niblack,J.F., and Gunsalus,I.C., (1965). Bacteriol. Proc., pp.115.

DISCUSSION

In characterising the two phages it was evident that neither phage 16 nor phage B was specific for one species since both were able to infect Pseudomonas fluorescens and Pseudomonas aeruginosa. Although some of the phages isolated in this laboratory appear to be specific for one species, it is not unusual for a pure phage strain to be virulent for more than one species or, for a different but closely related genera (Adams and Wade, 1954). The results obtained of course rule out the possibility of using phage 16 and phage B as a taxonomic tool to differentiate between Pseudomonas fluorescens and Pseudomonas aeruginosa.

Conversely, the fact that the two phage were virulent for the same strains of the two species (table 3) meant that the host range could not be used to characterize the two phage.

Differentiation of the phage was achieved by comparison of the plaque morphology. Of the two phage, phage B produced larger more distinct plaques than phage 16. It was noticed however, that the plaques produced by both phage varied in size (plates 1 and 2). Albeit no adsorption rate studies were done, it would seem from the variation in plaque size that phage 16 adsorbs slowly to its host. The rationale for this is that if a phage adsorbs early in the formation of the bacterial lawn a large plaque will result but if a phage does not infect until late in the developement

of the bacterial lawn a small plaque will result. Sagik (1954) found that E.coli phage, T2, produced poor plaques when it adsorbed slowly, Wahl and Blum-Emerique (1952) have shown that a phage which adsorbed slowly produced a variation in the size of plaques.

In the one step growth experiment an adsorption time of 10 minutes was used. The time allowed for adsorption will be limited by the necessity of completing the dilution before the end of the latent period. This presented no problem in these experiments since the latent period was approximately 45 minutes. If the adsorption period is prolonged however, initiation of infection is spread over a longer period and consequently the rise period will also be prolonged. The choice of 10 minutes seemed to best satisfy these two criteria.

The latent period of phage 16 was 45 minutes with a multiplicity of infection of one and 60 minutes with a multiplicity infection of ten. It is known that the length of the latent period should not be affected by the number of phage particles with which a host has been infected (Delbruck and Luria, 1942). In the experiments conducted unadsorbed phage were not inactivated and the apparent difference in the latent period with the varying multiplicities of infection is probably due to the free phage masking the early release of phage.

With a multiplicity of infection of one, phage 16 had a burst size of 35. Using a multiplicity of infection of ten, phage 16 had a burst size of approximately 100 and phage B had a burst size of approximately 60.

The higher burst size values are similar to values published in the literature for some of the T phage of E.coli (Ellis and Debruck, 1939; Doerman, 1959) and for phage 2 infectious for P. aeruginosa (Grogan and Johnson, 1964c). In the experiments conducted with phage 16 and phage B free phage were not inactivated and the values obtained will be only approximate. The difference in value between the lower and higher multiplicities of infection with phage 16 is probably due to the fact that a larger number of susceptible bacteria were infected at the higher multiplicity. Even though these values are approximate they appear to be in agreement with the values found for other phage.

The rise period for phage 16 and phage B was approximately 60 minutes. This is longer than the rise period of 10 minutes for some of the coliphage (Anderson and Doerman, 1952; Doerman, 1952) and phage 7S, an RNA phage infectious for P. aeruginosa (Feary etal, 1964). The value is similar however to the value obtained by Ellis and Delbruck (1939) in their original one step growth experiment with a phage infectious for E.coli.

It is possible that a shorter adsorption time should be used in the one step growth experiments with phage 16 and phage B to shorten the rise period.

Another characteristic of phage 16 and phage B is the increase in turbidity of the infected culture. With a multiplicity of infection of ten the increase in

turbidity of the infected culture parallels that of the uninfected culture. Feary etal (1964), using phage 7S, obtained complete clearing of the bacterial culture with a multiplicity slightly greater than one. Phage T4 of E.coli also cause clearing of the infected culture (Doerman, 1952) at a low multiplicity of infection.

Failure to cause clearing of the bacterial culture, the long rise period and the relatively long latent period are probably characteristic of the phages studied and may in fact be characteristic of most DNA phage infectious for pseudomonas. Whether adsorption is the limiting factor or something more subtle such as intracellular phage production is not known but the two phage, phage B and phage 16, seem to be "less virulent" for their host than do the T even phage of E.coli.

Purification of phage 16 on a DEAE cellulose column resulted in a preparation with a degree of purity comparable to that obtained on a preformed CsCl gradient. The phage was found to elute from the column at a NaCl concentration of 0.2M with 35% to 90% of the infectious phage being recovered. The O.D.230/O.D.260 ratio was 0.85 and the O.D.260/O.D.280 ratio was 1.52.

Murphy and Philipson (1962) obtained a two-fold increase in the purity of a phage infectious for Bacillus megatherium on a DEAE cellulose column. Using a procedure similar to that used for phage 16 they found that the phage were eluted from the column at a NaCl concentration of 0.12M or 0.14M with 30% to 50% of the infectious phage being recovered. The O.D.230/O.D.260

ratio was 0.85 and the O.D.260/O.D.280 ratio was 1.57. These results compare quite favorably with those obtained for phage 16.

Column chromatography on DEAE cellulose was of no use in purifying phage B since the phage did not adsorb on the column and less than 1% of the infectious phage were recovered. Electron micrographs indicate that passage of phage B through the column destroys the phage. Creaser and Taussig (1957) report the purification of E.coli phage, T2, on ECTEOLA. When this was tried with phage B it also proved unsuccessful.

Phage 16 had a buoyant density in a preformed CsCl gradient of 1.502gm.cm.$^{-3}$. This agrees with the value of 1.502 gm.cm.$^{-3}$ obtained by centrifugation to equilibrum in CsCl. (Yamamoto, 1964). This agreement is not entirely unexpected since Vinograd (1963) mentions that in experiments with viruses, globular proteins or globular particles, a preformed gradient can be used to shorten the time required to attain equilibrum. A preformed CsCl gradient was used by Mathews (1960) while working with Tobacco Yellow Mosaic Virus and a preformed RbCl gradient was used by Rueckrut (1962) to purify ØX-174.

Since DNA has an average buoyant density of 1.7 gm.cm.$^{-3}$in CsCl and protein has an average buoyant density of 1.3 gm.cm.$^{-3}$in CsCl (Weigle, 1959), a value of 1.50 gm.cm.$^{-3}$ would indicate that phage 16 contains approximately 50% DNA.

Weigle (1959) found that E. coli phage, , had a buoyant density in CsCl of 1.508 gm.cm.$^{-3}$ and he concluded that this represented approximately 50% DNA.
Murphy and Philipson (1962) found that Bacillus megatherium phage G had a buoyant density in CsCl of 1.50 gm.cm.$^{-3}$. By separate analysis they found that the phage contained 45% to 55% DNA, with the latter figure probably being more accurate.

Recently Niblack and Gunsalus (1965) reported a phage infectious for Pseudomonas putida which appeared morphologically identical to phage 16. The phage, designated as Pf, has a 54 mu diameter, polyhedral head and a 10 mu conical tail which is very similar to phage 16 (Yamamoto, 1964). The buoyant density of phage Pf in CsCl is 1.48 gm.cm.$^{-3}$, which is also similar to that found for phage 16. The percentage of DNA in phage Pf was not reported.

In calculating the molecular weight of phage 16, introduction of the buoyant density in place of the dry density, for calculation of the particle weight, will result in a value for the molecular weight which will be large due to hydration of the phage particle. However, since the radius of the phage is cubed in the equation for determining the particle weight of the phage, the error introduced in substituting the buoyant density for the dry density will be negligible in contrast to an error introduced in the measurement of the phage particle. As previously mentioned treatment of the phage preparation for electron microscope observation will result in some shrinkage of the phage particle. Consequently the smaller

diameter will result in a somewhat small molecular weight value for the phage.

The DNA isolated from phage 16 and P. fluorescens had a minimum absorbance at 231 mu and a maximum absorbance at 260 mu. The O.D.230/O.D.260 ratio was approximately 0.45 and the O.D.260/O.D.280 ratio was approximately 1.94. These values compare favourably with the values obtained for the salmon sperm DNA used as a control (table 6). The slight discrepancy is probably due to the small amount of protein which cannot be removed by phenol treatment (Mandel and Hershey, 1960).

Marmur (1961) reports that the average O.D.230/O.D.260 and O.D.260/O.D.280 ratios for DNA isolated from bacteria are 0.45 and 1.94 respectively. Zelle and Hollander (1954) show a typical absorption spectrum for DNA isolated from phage T2 in which the O.D.230/O.D.260 ratio is 0.43 and the O.D.260/O.D.280 ratio is 1.96.

The DNA of phage 16 contains adenine, thymine, cytosine and guanine. Chemical analysis indicates that there is 42.8 mole percent guanine plus cytosine. The DNA contains no glucose or unusual bases.

The appearance of a fifth but faint ultraciolet absorbing spot due to formic acid hydrolysis of DNA has been reported by Vischer and Chargaff (1948), Smith and Markham (1950) and Markham (1955). Although these references do not specifically state that deamination of cytosine is occuring Fu and Bojarska (1965) have shown that methyl cytosine is deaminated to form thymine. Thus the uracil

found in the phage 16 and pseudomonas DNA is probably formed by the deamination of cytosine.

Of the other pseudomonas phage studied one contains RNA (Feary etal 1964) and two others contain DNA (Grogan and Johnson, 1964; Niblack and Gunsalus, 1965). The phage studied by Grogan and Johnson, phage 2, contains the four usual bases adenine, thymine, cytosine, and guanine and has 54.5 mole percent guanine plus cytosine. (Grogan and Johnson, 1965). The phage characterized by Niblack and Gunsalus also contains the four normal bases but has a guanine plus cytosine content of 62 mole percent. (Niblack and Gunsalus, 1965).

It appears that the three DNA phage for pseudomonas studied to date are like the T odd phage of E.coli since no unusual bases or glucose are present. Phage 16 differs markedly from phage Pf and phage 2 in that the guanine plus cytosine content is only 43 mole percent. Phage 16 is analogous to phage T5 in this one respect. While the other T odd phage have a guanine plus cytosine content of approximately 49 mole percent, T5 has a guanine plus cytosine content of only 39 mole percent. The guanine plus cytosine content of T5 and phage 16 therefore differ markedly from the guanine plus cytosine content of their respective hosts. On the other hand the guanine plus cytosine content of phage 2, Pf and the other T odd phage approaches the guanine plus cytosine content of their respective hosts.

Phage 16 DNA had a buoyant density of 1.702 gm.cm.$^{-3}$

in a CsCl gradient. By calculation (Schildkraut etal, 1962) this corresponds to 43 mole percent guanine plus cytosine. From the standard curve (figure 16) this is found to be equal to 44 mole percent guanine plus cytosine. Since the two values are equal to the chemically determined value it is unlikely that there are any unusual bases and/or glucose. The only double stranded DNA's which have higher buoyant densities in CsCl than is expected from the chemically determined base composition are those of the T even series of E.coli phage (Schildkraut etal, 1962) and some Bacillus subtilis phage (Takahashi and Marmur, 1963; Kallen etal, 1962). The anomalous buoyant density in the case of the T even phage is attributable to the presence of 5-hydroxymethylcytosine (Wyatt and Cohen, 1952) and/or glucose (Sinsheimer, 1954; Volkin, 1954; Jesaitus, 1956) in the DNA molecule. Phage SP8, infectious for Bacillus subtilis contains 5-hydroxymethyluracil (Kallen etal, 1962) and phage PBS-2 also infectious for Bacillus subtilis has uracil and glucose (Takahashi and Marmur, 1963) in the DNA molecule.

The DNA of P. fluorescens was found by chemical measurement to contain 64.7 mole percent guanine plus cytosine. The buoyant density of the DNA in CsCl was found to be 1.725 $gm.cm.^{-3}$. By calculation (Schildkraut etal, 1962) this corresponds to 63.8 mole percent guanine plus cytosine. From the standard curve a value of 65 mole percent guanine plus cytosine is obtained. These values are not in the range of 60 to 62 mole percent guanine plus

cytosine published by Marmur etal (1963) but do agree with earlier values of approximately 63.5 mole percent guanine plus cytosine (Lee etal, 1956; Catlin and Cunningham, 1958).

BIBLIOGRAPHY

Adams, M.H., (1959). Bacteriophages, Interscience, N.Y.

Adams, M.H., and Wade, E., (1954) Classification of Bacterial Viruses: The Relationship of Two Serratia Phages to Coli-dysentry Phages T3, T7 and D44. J. Bacteriol., 68, 320.

Allen, R.J.L., (1940). The Estimation of Phosphorus. Biochem. J., 34, 858.

Bendich, A., (1957). Methods For Characterization of Nucleic Acids by Base Composition in "Methods in Enzymology". (S.R.Colowick and N.O.Kaplan eds). Vol. 3, pp 715, Academic Press Inc., N.Y.

Cancik, J., (1923). Über Pyozyaneusbakteriophagen. Časopis ces lek., 62, 183.

Catlin, B.W., and Cunningham, L.S. (1958) Studies of Extracellular and Intracellular Bacterial Deoxyribonucleic Acids. J.Gen.Microbiol., 19,522.

Chargaff, E., Lipshitz, R., and Green, C., (1952). Composition of the Deoxypentose Nucleic Acids of Four Genera of Sea-Urchin. J.Biol.Chem., 195, 155.

Combiesco, D., and Margheru, A., (1923). Sur la lyse du Bacille pyocyanique. C.R.Soc.Biol., 88, 912.

Creaser, E.H., and Taussig, A., (1957). The Purification and Chromatography of Bacteriophages on Anion Exchange Cellulose. Virology, 4, 200.

Delbrück, M., (1940). The Growth of Bacteriophages and Lyses of the Host. J.Gen.Physiol., 23, 643.

Delbrück, M., and Luria, S.E., (1942). Interference Between Two Bacterial Viruses Acting Upon the Same Host and Mechanism of Virus Growth. Arch. Biochem., 1, 111.

Dische, Z., (1955). Color Reactions of Nucleic Acid Components in "The Nucleic Acids". (E.Chargaff and J.N.Davidson eds.) Vol. 1, Academic Press Inc., N.Y.

Doermann, A.H., (1952). The Intracellular Growth of Bacteriophages. 1: Liberation of Intracellular Bacteriophage T4 by Premature Lysis With Another Phage or With Cyanide. J. Gen. Physiol., 35, 645.

Ellis, E.L., and Delbrück, M., (1939) The Growth of Bacteriophage. J.Gen.Physiol., 22, 365.

Erikson, R.L., and Szybalski, W., (1964) The Cs_2 SO_4 Equilibrium Density Gradient and its Application for the Study of T-even Phage DNA: Glucosylation and Replication. Virology, 22,111.

Fastier, L.D., (1945) A Bacteriophage for Pseudomonas pyocyanea. J.Bacteriol., 50, 301.

Feary, T.W., Fisher, E., and Fisher, T.N., (1963a). Lysogeny and Phage Resistance in Pseudomonas aeruginosa. Proc. Soc. Exptl.Biol.Med. 113, 426.

Feary, T.W., Fisher, E., and Fisher, T.N. (1963b). A Small RNA Containing Pseudomonas aeruginosa Bacteriophage. Biochem.Biophys.Res.Commun.,10,359.

Feary, T.W., Fisher, E., and Fisher, T.N., (1964) Isolation and Preliminary Characteristics of Three Bacteriophages Associated with a Lysogenic Strain of Pseudomonas aeruginosa. J. Bacteriol., 87, 196.

Fu, S.C.J., and Bojarska, A., (1965). Stability of 5-Methylcytosine During Acid Hydrolysis. Fed. Proc., 24, 2976.

Grogan, J.B., and Johnson, E.J. (1964,a). Phage Host Relationship and Physical Characteristics of a Pseudomonas aeruginosa Bacteriophage. J. Bacteriol., 87, 973.

Grogan, J.B., and Johnson, E.J. (1964,b). Nucleic Acid Composition of a Pseudomonas aeruginosa Bacteriophage. Virology, 24, 235.

Hadley, P., (1924). Transmissible Lysis of Bacillus pyocyaneus. J. Infect.Dis., 34, 260.

Hershey, A.D., Kalmanson, G., and Bronfenbrenner, J., (1943). Quantitative Methods in the Phage-Antiphage Reaction. J. Immunol., 46, 267.

Holloway, B.W., (1960,a). Grouping Pseudomonas aeruginosa by Lysogeny and Pyocinogenicity. J. Path. Bacteriol., 80, 448.

Holloway, B.W., Egan, J.B., and Monk, M., (1960,b). Lysogeny in Pseudomonas aeruginosa. Aust. J. Exptl. Biol., 38, 321.

Jesaitus, M.A., (1956). Differences in the Chemical Composition of the Phage Nucleic Acids. Nature, 178, 637.

Kallen, R.G., Simon, M., and Marmur, J., (1962) The Occurence of a New Pyrimidine Base Replacing Thymine in Bacteriophage DNA: 5-Hydroxymethyluracil. J.Mol.Biol., 5, 248.

Lee, Ki Yong, Wahl, R., and Barbu, E., (1956). Contenu en Bases Puriques et Pyrimidiques des Acides Désoxyribonucléiques des Bacteries. Ann. Inst. Pasteur., 91, 212.

Mandell, J.D., and Hershey, A.D., (1960). A Fractionating Column For the Analysis of Nucleic Acids. Analy. Biochem., 1, 66.

Markham, R., (1955). Nucleic Acids. Their Components and Related Compounds. Moderne Methoden Der Pflanzenanalyse, 4, 245.

Marmur, J., (1961). A Procedure For the Isolation of Deoxyribonucleic Acid From Micro-organisms. J. Mol. Biol., 3, 208.

Marmur, J., Falkow, S., and Mandel, M., (1963). New Approaches to Bacterial Taxonomy. Ann. Rev. Microbiol., 17, 329.

Mathews, R.E.F., (1960). Properties of Nucleoprotein Fractions Isolated From Turnip Yellow Mossaic Virus Preparations. Virology, 12, 521.

Meselsohn, M., Stahl, F.W., and Vinograd, J., (1957) Equilibrium Sedimentation of Macromolecules in Density Gradient. Proc. Natl. Acad. Sci. U.S., 43, 501.

Murphy, J.S., and Philipson, L., (1962). Purification of B. megatherium Phage B and Evidence For a Muralytic Enzyme as an Integral Part of the Phage. J. Gen. Physiol., 45, 155.

Niblack, J.F., and Gunsalus, I.C., (1965). Characterization of the Pseudomonas putida Bacteriophage Pf. Bacteriol. Proc., pp 115.

Pons, R., (1923). Bactériophage du pyocyanique. C. R. Soc. Biol., 89, 77.

Rabinowitz, G., (1934,a). Studies on Bacteriophage. 1. Relation of Bacteriophage and Other Lytic Agents to Negative Stool Cultures. Study of Two Cases. J. Bacteriol., 28, 221.

Rabinowitz, G., (1934,b). Studies on Bacteriophage. 11. Comparison of Action of Lytic Agents and Bacteriophage Upon pyocyaneus Cultures. J. Bacteriol., 28, 237.

Rolfe, F., and Meselsohn,M., (1959). The Relative Homogeneity of Microbial DNA, Proc. Natl. Acad. Sci. Wash., 45, 1039.

Rueckirt, R.R. Zillig, W., and Huber, K., (1962). An Improved Procedure for the Purification of Bacteriophage ØX-174. Virology, 17, 204.

Sagik, P., (1954). A Specific Reversible Inhibition of Bacteriophage T2. J. Bacteriol., 68, 430.

Schildkraut, C.L. Marmur, J., and Doty, P., (1962). Determination of the Base Composition of Deoxyribonucleic Acid From its Buoyant Density in CsCl. J. Mol. Biol., 4, 430.

Sinsheimer, R.L., (1954). Nucleotides from $T2r^{+}$ Bacteriophage. Science, 120, 551.

Sinsheimer, R.L., (1960). The Nucleic Acids of the Bacterial Viruses in "The Nucleic Acids". (E.Chargaff and J.N.Davidson eds.) Vol. 3, Academic Press Inc., N.Y.

Smith, I., (1960) Chromatographic and Electrophoretic Techniques, Vol. 1, pp.237, Interscience Publishers Inc..

Stent, G.S., (1963). Molecular Biology of Bacterial Viruses, W.H.Freeman and Company.

Sueoka, N., Marmur, J., and Doty, P., (1959). Heterogeneity in Deoxyribonucleic Acids. 11: Dependence of the Density of Deoxyribonucleic Acids on Guanine Plus Cytosine Content. Nature, 183, 1429.

Takahashi, I., and Marmur, J., (1963). Glucosylated DNA From a Transducing Phage for Bacillus subtilis. Biochem. Biophys. Acta. Comm., 10, 289.

Vinograd, J., (1963). Sedimentation Equilibrium in a Buoyant Density Gradient in "Methods in Enzymology" (S.P.Colowick and N.O.Kaplin eds.) Vol. 6, Academic Press Inc., N.Y.

Volkin, E., (1954). The Linkage of Glucose in Coliphage Nucleic Acids. J. Am. Chem. Soc., 76, 5892.

Warner, P.T.J.C.P., (1950). The Isolation of the Bacteriophage of Pseudomonas pyocyanea. Brit. J. Exp. Path., 31, 112.

Weigle, J.J., Meselsohn, M., and Paigen, K., (1959). Density Alterations Associated with Transducing Ability in the Bacteriophage . J. Mol. Biol., 1, 379.

Wyatt, G.R., and Cohen, S.S. (1952). A New Pyrimidine Base from Bacteriophage Nucleic Acids. Nature, 170, 1072.

Wyatt, G.R., and Cohen, S.S., (1953). The Bases of the Nucleic Acids of Some Bacterial and Animal Viruses. 1. The Occurence of 5-Hydroxymethylcytosine. Biochem.J., 55,774.

Yamamoto, T., (1964). Personal Communication.

Zelle, M., and Hollander, A., (1954). Monochromatic Ultraviolet Absorbtion Spectra and Quantum Yields for Inactivation of T1 and T2 Escherichia coli Bacteriophages. J. Bacteriol., 68, 210.

Anderson, T.F., and Doermann, A.H., (1952). The Intracellular Growth of Bacteriophages (II) The Growth of T3 Studied by Sonic Disintegration and by T6 Cyanide Lysis of Infected Cells. J. Gen. Physiol., 35, 657.

Smith, J.D., and Markham, R., (1950). Chromatographic Studies on Nucleic Acids. Biochem. J., 46, 509.

Vischer,D., and Chargaff, E., (1948). The Composition of the Pentose Nucleic Acids of Yeast and Pancreas. J. Biol. Chem., 176, 715.